BACTERIAL PRODUCTION OF POLY-γ-GLUTAMIC ACID AND EVALUATION OF ITS EFFECT ON THE VIABILITY OF PROBIOTIC MICROORGANISMS

A thesis submitted for the degree of

Doctor of Philosophy

By Aditya Bhat, MSc

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is a naturally occurring biopolymer made up of repeating units of glutamic acid and can be potentially used for multiple applications. This study compared the production of γ-PGA by eight bacteria (*B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858 *B. subtilis* 23859, *B. subtilis* natto, *B. licheniformis* 1525, *B. licheniformis* 6816 and *B. licheniformis* 9945a) in GS and E media. *B. subtilis* natto and *B. licheniformis* 9945a have been investigated extensively for γ-PGA production, however, the remaining six have not previously been used. Using the eight bacteria, yields of up to 22.3 g/l were achieved in shake flasks. On characterization, it was observed that γ-PGA with different properties (crystallinity, acid/salt form and molecular weights ranging from 3,000 Da to 871,000 Da) was produced. Production of γ-PGA by *B. subtilis* natto in GS medium was scaled up using a fermenter and was tested for novel probiotic applications. The survival of probiotics during freeze drying, storage and ingestion was improved by combining them with a γ-PGA matrix. For *L. paracasei*, 10% γ-PGA protected the cells significantly better ($P < 0.05$) than 10% sucrose during freeze drying, whereas for *B. longum* and *B. breve*, it showed comparable cryoprotectant activity ($P > 0.05$) to 10% sucrose. This study also demonstrated the potential use of a non-dairy foodstuff (orange juice) for delivery of probiotics. Two *Bifidobacteria* strains protected with γ-PGA survived significantly better ($P < 0.05$) in orange juice for 39 days, with a log reduction in viability of less than 2.99 CFU/ml, when compared to unprotected cells, which showed complete loss in viability by day 20. In addition, γ-PGA protection improved survival of *Bifidobacteria* in a solution mimicking the environment of the stomach. γ-PGA-protected *Bifidobacteria* showed little (< 0.47 log CFU/ml) or no loss in viability when stored in simulated gastric juice (pH 2.0) for four hours, whereas unprotected cells died within two hours.

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For my parents, Anand, Ajit, Nisha and Noor

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ABBREVIATIONS

Acquired Immunodeficiency Syndrome	AIDS
Bacterial vaginosis	BV
Bifidus Selective Medium	BSM
Cis-dichlorodiammineplatinum (II)	CDDP
Colony Forming Units	CFU
Cross linked γ-PGA	PGA (XL)
deMan Rogosa Sharpe	MRS
Dilution Factor	DF
Dimethyl sulphoxide	DMSO
Diphenhydramine	DPH
Dissolved oxygen	dO_2
Food and Agriculture Organization	FAO
Food and Drug Administration	FDA
Fructooligosaccharide	FOS
Fourier Transform Infra-Red Spectroscopy	FT-IR
Gel Permeation Chromatography	GPC
Helicobacter pylori	HP
Hemagglutinins	HA
High Performance Liquid Chromatography	HPLC
Human Immunodeficiency Virus	HIV
Hydrophobic derivatives of γ-PGA	γ-hPGA
Inductively Coupled Plasma – Atomic Emission Spectroscopy	ICP-AES
L-phenylalanine ethylester	L-PAE
Lactic Acid Bacteria	LAB
Molecular weight	M_w
Molecular number	M_n
National Collection of Industrial and Marine Bacteria	NCIMB
Natural Killer	NK

Partial pressure of oxygen	pO_2
Polydispersity	Pd
Phosphate Buffered Saline	PBS
Peptide DNA	pDNA
Polyethyleneimine	PEI
Polyglutamic acid	PGA
Poly-α-glutamic acid	α-PGA
Poly-γ-glutamic acid	γ-PGA
Polyhydroxyalkanoates	PHA's
Respiratory Tract Infections	RTI
Scanning Electron Microscopy	SEM
Transmission Electron Microscopy	TEM
Trypticase-Phytone-Yeast extract	TPY
Tryptone Soya Agar	TSA
Tryptone Soya Broth	TSB
Ulcerative Colitis	UC
Urinary Tract Infections	UTI
Vulvovaginal candidiasis	VVC
World Health Organization	WHO
X-Ray Diffraction	XRD

1. INTRODUCTION – POLY-γ-GLUTAMIC ACID

1.1 INTRODUCTION TO γ-PGA

Polyglutamic acid (PGA) is a biodegradable, non-immunogenic and anionic homopolyamide that is made up of D and L glutamic acid units (Shih and Van, 2001).

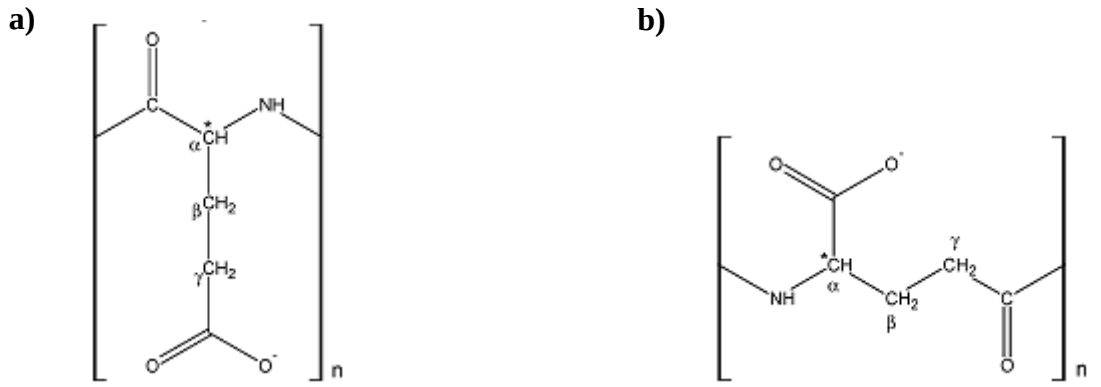


Fig 1.1: a) Structure of α-PGA where individual glutamic acid residues are connected to each other by amide linkages between α-amino and α-carboxylic acid groups b) Structure of γ-PGA where individual glutamic acid residues are connected to each other by amide linkages between α-amino and γ-carboxylic acid groups. (Buescher and Margaritis, 2007)

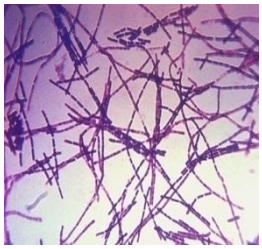
It is protein-like, but while proteins have more than one type and a fixed number of amino acids, PGA is made up of only glutamic acid residues and does not have a fixed molecular weight (Shi *et al.*, 2007). PGA can be differentiated into 2 isoforms – poly-α-glutamic acid (α-PGA) and poly-γ-glutamic acid (γ-PGA) - depending on the attachment of the amino group to the carboxyl group (**Fig 1.1**). α-PGA is synthesized chemically by nucleophile initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid (Buescher and Margaritis, 2007). Microbial production of α-PGA is difficult and the polymer can only be produced by recombinant technology (Buescher and Margaritis, 2007). γ- PGA has been produced extensively using bacteria, especially those of *Bacillus* sp. It is different from other proteins, because inside the cell, glutamate is polymerized via the γ-amide linkages, and thus is synthesized in a ribosome independent manner (Bodnár *et al.*, 2008; Akagi *et al.*, 2007). Hence, substances that can inhibit translation of proteins, such as chloramphenicol, have no effect on the production of γ-PGA. Due to the γ-linkage of its component glutamate residues,

γ-PGA is resistant to α-proteases, since α-proteases cleave α-amino linkages (Candela and Fouet, 2006).

In their review paper on PGA, Buescher and Margaritis (2007) have pointed out that α-PGA and γ-PGA have sometimes been confused with each other (Buescher and Margaritis, 2007). Section 1 provides a brief updated review on the production, properties, mechanism of synthesis and applications of microbially produced γ-PGA.

1.2 PRESENCE OF γ-PGA IN NATURE

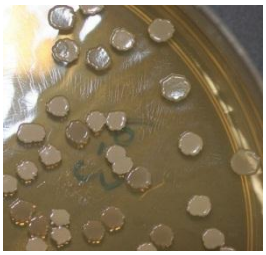
Some of the natural sources of γ-PGA can be seen in **Fig 1.2**. γ-PGA was first discovered by Ivonovics and co-workers in 1937 when the capsule of *Bacillus anthracis* was released into the medium upon autoclaving or upon aging and autolysis of the cells (Shih and Van, 2001). Another naturally occurring source of γ-PGA is the mucilage of natto and chungkookjang, traditional foods in Japan and Korea respectively, that contain soybeans fermented with *Bacillus subtilis* natto and *Bacillus subtilis* subsp. chungkookjang (Shih and Van, 2001; Candela and Fouet, 2006; Park *et al.*, 2005). γ-PGA is produced by some Gram-positive bacteria (such as *B. subtilis* and *B. licheniformis*), some archaea and some eukaryotes (Weber, 1990; Hezayen *et al.*, 2001). No Gram-negative bacteria were known to produce the polymer, until the work done by Candela *et al.* (2009), which demonstrated that *Fusobacterium nucleatum* produces γ-PGA (Candela *et al.*, 2009). Efforts have been made to insert the genes responsible for γ-PGA production into *E. coli* and plants such as tobacco, to gain more knowledge regarding the molecular mechanism for γ-PGA production as well as increase yield while reducing cost of production (Ashiuchi *et al.*, 1999; Tarui *et al.*, 2005). γ-PGA has also been found covalently linked to tubulin in neurons of mice (Edde *et al.*, 1990).



B. anthracis
(Produces membrane-bound γ-PGA)

Natto
(Japanese food item)

B. licheniformis
(Produces γ-PGA extracellularly)



B. subtilis
(Produces γ-PGA extracellularly)

Hydra
(Present in nematocysts)

Mice
(Present in neurons)

Fig 1.2: Some examples of γ-PGA in nature

1.3 FUNCTION OF γ-PGA

The function of γ-PGA depends on the organism producing it and the environment the organism inhabits. For instance, presence of γ-PGA has been shown in neurons of mice where it is thought to play a role in the regulation of microtubule dynamics by modifying the interaction of tubulin with tubulin-associated proteins and calcium (Edde *et al.*, 1990). *Cnidaria* are also known to produce γ-PGA. These animals have nematocysts (stinging cells) which are used for prey capture, for locomotion and for protection by an explosion mechanism. Large amounts of γ-PGA help to trigger this explosion by creating an osmotic pressure (Weber, 1990). In other organisms producing γ-PGA, the function depends on whether it is membrane bound or released into the environment.

1.3.1 Membrane bound γ-PGA

If γ-PGA is membrane bound, it may act as a source of glutamate when bacteria are starved in late stationary phase (Kocianova *et al.*, 2005; Kimura *et al.*, 2004b). Glutamate is a source of nitrogen and can also be used for conversion into other amino acids and Krebs cycle intermediates. In addition, it has been shown that the capsules of virulent strains of *B. anthracis* contain solely γ-D-PGA (Tomcsik and Szongott, 1933 in Candela and Fouet, 2006). The D enantiomer in *B. anthracis* capsule makes the bacterium non-immunogenic (Zwartouw and Smith, 1956). The γ-PGA protects the bacterial cells against phage infections and also prevents antibodies from gaining access to the bacterium (Mesnage, 1998). Likewise, *Staphylococcus epidermidis* also synthesize surface associated γ-PGA (Kocianova *et al.*, 2005). The γ-PGA protects *Staphylococcus epidermidis* against antimicrobial peptides (Kocianova *et al.*, 2005). In both cases, γ-PGA helps the bacteria to escape phagocytosis, hence contributing to virulence. In addition, since γ-PGA has non-peptide bonds, it is not cleaved by common proteases. This provides protection to cells producing the polymer from various cell extracellular enzymes that cleave cell wall associated proteins (Kaplan, 1998).

1.3.2 Released γ-PGA

Unlike *B. anthracis*, some of the soil bacteria release γ-PGA into the environment after production, where it can help them survive adverse conditions (McLean *et al.*, 1990; Kimura *et al.*, 2004b). Bacteria use the released γ-PGA for the sequestration of toxic metal ions, increasing their resistance to challenging environments (McLean *et al.*, 1990). *Planococcus halophilus*, *Sporosarcina halophila* and *Natrialba aegyptiaca* specifically use γ-PGA to decrease high local salt concentrations (Hezayen *et al.*, 2001; Kandler *et al.*, 1983). It is thought that some bacteria such as *B. licheniformis* produce extracellular γ-PGA as an alternative to sporulation for cell survival (Kaplan, 1998).

1.4 MECHANISM OF γ-PGA PRODUCTION

One of the major challenges of making γ-PGA usage applicable in industry is reducing the cost of its production. It has been estimated that the cost of using γ-PGA is several tens to hundred fold more expensive than the conventional materials it could replace (Sung *et al.*, 2005). Reducing the cost of production is the only foreseeable solution to this problem. Designing mass production systems for γ-PGA would be a major step towards a potential solution. To achieve this, the knowledge of how different factors affect the yield of production needs to be obtained. Information about genes and enzymes involved in γ-PGA production would certainly help in manipulating organisms for more efficient production of γ-PGA. Also, γ-PGA production in bacteria can change unexpectedly, even if strict conditions are maintained (Bajaj and Singhal, 2011). Therefore, it becomes all the more important to identify the factors involved in γ-PGA production on a molecular level and assess factors affecting its production.

Many researchers have investigated the genetics of γ-PGA in the last decade (Sung *et al.*, 2005; Buescher and Margaritis, 2007; Candela and Fouet, 2006; Ashiuchi and Misono, 2002) and genes that play a role in every step of γ-PGA production have been identified. Therefore, a brief understanding of the current probable aspects of the mechanism of γ-PGA synthesis is provided in this section.

The process of γ-PGA production can be seen to have 4 distinct branches – γ-PGA racemisation, γ-PGA polymerization, γ-PGA degradation and γ-PGA regulation. Each of these are discussed in the sections below.

1.4.1 γ-PGA racemisation

γ-PGA can be composed of only L, or only D, or a mixture of both L and D enantiomers of glutamic acid in varying amounts, depending on the bacterial strain producing it and the culture medium used for its production. Incorporation of D-glutamic acid into the growing chain of γ-PGA requires racemization of L-glutamic acid, which is either exogenously supplied or produced *de novo* by the bacterium (Shih and Van, 2001).

For *B. subtilis*, the genes responsible for coding the enzymes involved in racemisation are *glr* and *yrpC* (Ashiuchi and Misono, 2002; Buescher and Margaritis, 2007). Glr is a cytosolic enzyme with a high selectivity for glutamic acid and a preference for L-glutamic acid (Thorne *et al.*, 1955; Buescher and Margaritis, 2007). It was also shown that Glr formed D-glutamic acid only when the cells were grown on complex medium whereas YrpC was found to be active when the cells were grown on minimal medium (Kimura *et al.*, 2004a). In *B. anthracis*, *B. subtilis*, *B. licheniformis* and *S. epidermidis*, the chromosomal positions of *glr* and *yrpC* are a long distance (500 kB) from the glutamic acid synthesis genes. Hence, it seems unlikely that genes responsible for racemisation would be involved in γ-PGA synthesis (Buescher and Margaritis, 2007). Research has shown that Mn^{2+} affects the enantiomeric composition of γ-PGA (Cromwick and Gross, 1995a; Cromwick and Gross, 1995b; Perez-Camero *et al.*, 1999; Wu *et al.*, 2006) and it has been shown that Mn^{2+} affected the enantiomeric composition by altering the expression of the *glr* gene (Ashiuchi *et al.*, 2004).

A mechanism for racemization of L-glutamic acid to D-glutamic acid has been proposed by multiple researchers (Ho *et al.*, 2006a; Shih and Van, 2001). A diagrammatic representation of this mechanism can be seen in **Fig 1.3**. The enzyme L-glutamic acid : pyruvic acid

aminotransferase catalyzes the conversion of L-glutamic acid to L-alanine (1). L-alanine is then racemized into D-alanine using alanine racemase (2). D-alanine is then converted to D-glutamic acid using the enzyme D-glutamic acid : pyruvic acid aminotransferase (3). The L-glutamic acid and D-glutamic acid are then incorporated into the growing γ-PGA chain.

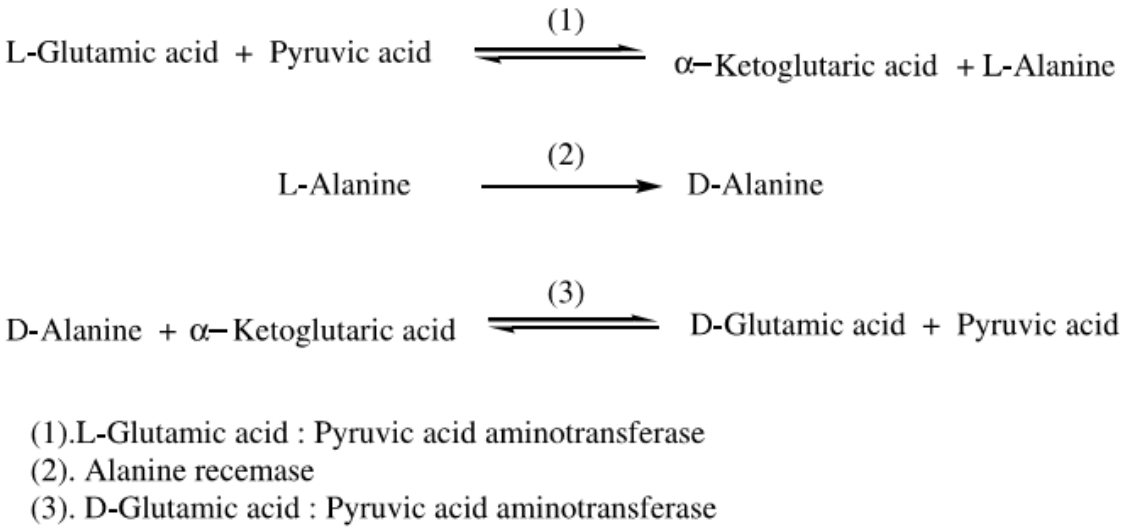


Fig 1.3: Conversion of L-glutamic acid to D-glutamic acid during synthesis of γ-PGA (Shih and Van, 2001)

Bacteria producing D-rich γ-PGA, such as *B. anthracis*, demonstrate a high D-amino acid aminotransferase activity. D-amino acid transferase is thought to be functionally insignificant in synthesis of γ-PGA from *B. subtilis*.

1.4.2 γ-PGA polymerization

In the case of *B. anthracis*, the genes involved in γ-PGA synthesis lie on a large plasmid, whilst in other *Bacillus* species, the genes are present on the chromosome (Shih and Van, 2001; Ashiuchi *et al.*, 2001).

When γ-PGA is surface associated or anchored (as in the case of *B. anthracis*), the "*cap*" (capsule) genes are required for its production. On the other hand, for γ-PGA that is released, the "*pgs*" (polyglutamate synthase) genes come into action (Candela and Fouet, 2006). Both the *cap* and *pgs* gene sets have at least 4 genes, designated the *cap* (or *pgs*) *B*, *C*, *A* and *E* (**Fig 1.4**). The *pgsBCA* genes of *B. subtilis* IFO3336 (*B. natto*) are homologous to *capBCA* genes of *B. anthracis* (Makino *et al.*, 1989; Shih and Van, 2001). The unique membrane bound PgsBCA complex in *B. subtilis* is highly unstable and hydrophobic which makes isolation of this complex challenging (Shih and Van, 2001). However, attempts have been made to understand its importance on γ-PGA production (Ashiuchi *et al.*, 1999; Sung *et al.*, 2005).

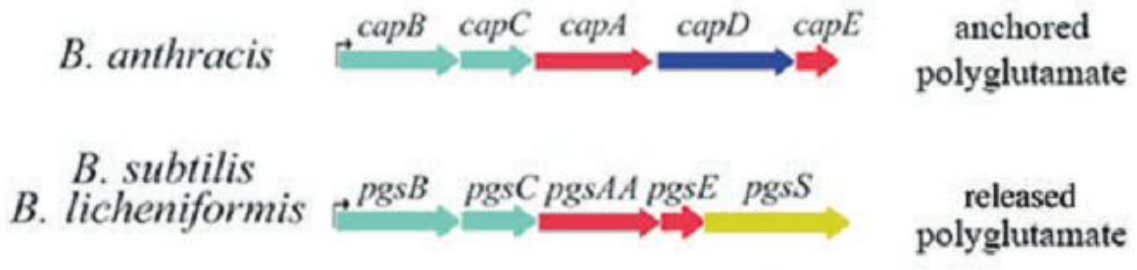


Fig 1.4: The genetic setup required for γ-PGA synthesis when it is anchored (*B. anthracis*) and when it is released (*B. subtilis* and *B. licheniformis*) (Candela and Fouet, 2006).

pgsBCA has been identified as the sole machinery responsible for γ-PGA synthesis in *Bacillus subtilis*, when it was shown that pgsBCA null mutants produced by disruption of pgsBCA genes in *B. subtilis* (chungkookjang) were incapable of γ-PGA production (Sung *et al.*, 2005). They showed that, even if one of the genes is absent, γ-PGA production is impossible. In contrast, Urushibata *et al.* (2002) are of the opinion that only *pgsB* and *pgsC* are required for the production of γ-PGA (Urushibata *et al.*, 2002a). These authors also found that strains that have *pgsBCA*, but which do not produce γ-PGA, do so because the genes are not translated, not because an inactive gene product is produced (Urushibata *et al.*, 2002b). Candela *et al.* (2005) showed that CapE (a 47 amino acid peptide) is also responsible and

essential for the production of γ-PGA (Candela *et al.*, 2005) as it appeared to interact with CapA.

The mechanism of polymerisation is shown to be dependent on ATP (**Fig 1.5**). The proposed reaction mechanism for γ-PGA synthesis (Sung *et al.*, 2005) is as follows: firstly, a terminal carboxyl group of the growing γ-PGA chain acts as an acceptor for phosphoryl group transfer from the gamma phosphate of ATP. Secondly, the amino group of glutamic acid acts as a donor for nucleophilic attack on the phosphorylated carboxyl group, resulting in the formation of an amide linkage and a γ-PGA chain elongated by one glutamic acid residue. This reaction continues to polymerize γ-PGA at the active site of the synthetase complex (PgsBCA).

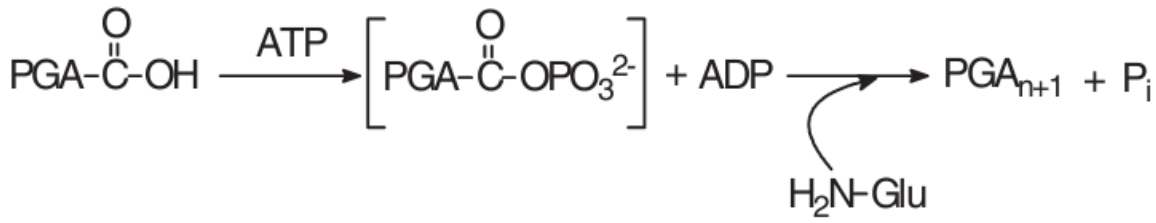


Fig 1.5: Reaction showing the dependence of γ-PGA polymerization on ATP (Sung *et al.*, 2005).

PgsB and PgsC together form the catalytic site whereas PgsA is involved in the removal of the elongated chain from the active site so that the next glutamic acid monomer can be added. Activity of PgsBCA is dependent on Mg^{2+} (Buescher and Margaritis, 2007). Transportation of γ-PGA outside the cell can be facilitated by a less compact cell membrane with shorter phospholipids (Buescher and Margaritis, 2007). γ-PGA can be anchored to the bacterial surface or released (**Fig 1.6**). CapD, a γ-glutamyl-transpeptidase, catalyses the anchorage of γ-PGA to peptidoglycan in *B. anthracis* (Candela and Fouet, 2006). On the other hand, PgsS is a hydrolase that catalyses the release of γ-PGA. The location of PgsS has not yet been

determined, but is assumed to be present on the surface of the cells (Candela and Fouet, 2006).

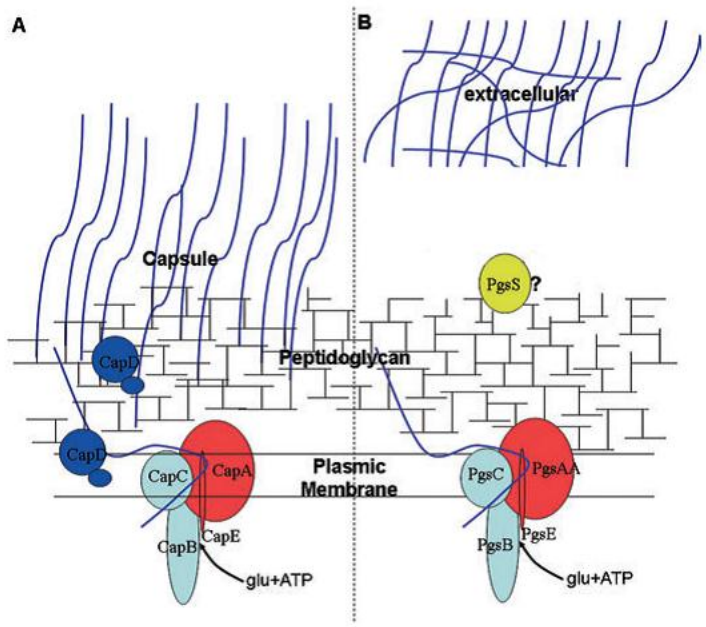


Fig 1.6: Schematic representation of γ-PGA synthesis membrane-anchored complex. A: γ-PGA is anchored covalently to the peptidoglycan in *B. anthracis* via CapD. B: γ-PGA is released in *B. subtilis* and *B. licheniformis* via PgsS. "?" represents lack of location certainty (Candela and Fouet, 2006).

A full biosynthetic pathway for the production of γ-PGA has been proposed (**Fig 1.7**). Synthesis of γ-PGA is thought to be a two-step process. The first step involves the production of L-glutamic acid and D-glutamic acid which is followed by polymerization of these glutamic acid residues to form γ-PGA (Bajaj and Singhal, 2009a). L-glutamic acid units that make up γ-PGA can be derived from two sources. It can either be obtained exogenously or endogenously via the glutamic acid biosynthetic pathway. Endogenous production of L-glutamic acid requires conversion of a carbon source via acetyl-CoA and TCA cycle intermediates. α-ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis (Ko and Gross, 1998; Rehm, 2009). Exogenous L-glutamic acid can be converted to L-glutamine with the help of the enzyme glutamine synthetase. L-glutamine

can be a precursor of γ-PGA because it can be involved in amino group transfer to α-ketoglutaric acid, forming two molecules of L-glutamic acid from one L-glutamine and one α-ketoglutaric acid molecule. These reactions are catalysed by L-glutamine synthetase and glutamine-2-oxoglutarate aminotransferase (Bajaj and Singhal, 2009a). When glutamine is not available, inorganic ammonia and α-ketoglutaric acid are used for L-glutamic acid production, involving the enzyme L-glutamic acid dehydrogenase (Bajaj and Singhal, 2009a). L-aspartic acid and α-ketoglutaric acid can also be converted to oxaloacetic acid and L-glutamic acid using L-aspartic acid aminotransferase. In addition, exogenous amino acids that belong to the glutamic acid family, such as L-arginine and L-proline can be imported into the cell and converted to L-glutamic acid, which can then be incorporated into the growing γ-PGA chain (Ashiuchi, 2010).

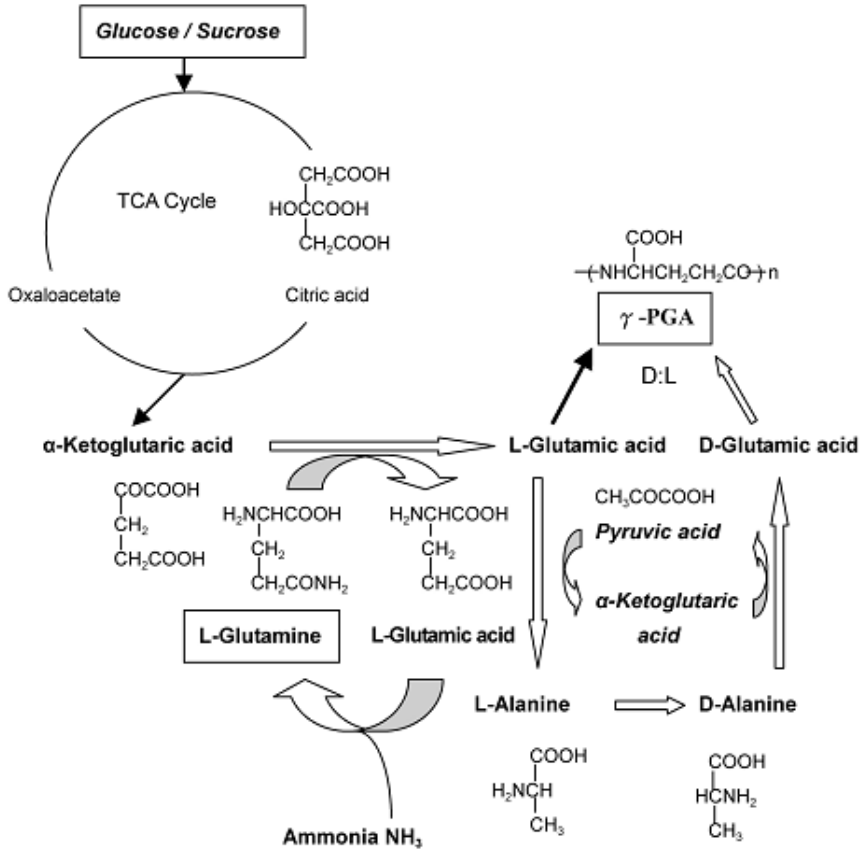


Fig 1.7: A proposed pathway for the synthesis of γ-PGA in *Bacilli*. TCA = Tricarboxylic acid cycle (Ho *et al.*, 2006a).

1.4.3 γ-PGA degradation

Two enzymes (endo-γ-glutamyl peptidase and exo-γ-glutamyl peptidase) have been shown to be associated with degradation of γ-PGA *in vivo*. The endo-γ-glutamyl peptidase coding genes – *ywtD*, *dep* or *pgdS* – are located downstream from and in the same orientation as the *pgsBCA* operon (Ashiuchi *et al.*, 2003; Buescher and Margaritis, 2007; Suzuki and Tahara, 2003). The exo-γ-glutamyl peptidase genes – *ggt* or *capD* are located some distance away from the *pgsBCA* complex in most *Bacilli* (Uchida *et al.*, 1993). However, in the case of *B. anthracis*, they are placed directly downstream of the *pgsBCA* complex. Recently, Yao *et al.* (2009) investigated the presence and activity of γ-PGA depolymerase enzyme in *B. subtilis* NX-2 which is responsible for the depolymerisation of γ-PGA in batch culture (Yao *et al.*, 2009). The enzyme was seen to be active extracellularly in the culture and was shown to be an endo-hydrolase. The gene encoding the enzyme was *ywtD*. The YwtD protein was obtained in purified form after the gene was cloned and expressed in *E. coli*. The enzyme was active between a temperature range of 30-40°C and a pH range of 5-8. At the optimal pH and temperature (5 and 30°C respectively), a reduction in the molecular weight of γ-PGA (from 1000 – 20 kDa) was observed. Polydispersity decreased as a function of depolymerization time. The enzyme was also seen to be active extracellularly during the late stationary phase. When compared with physical and chemical methods of γ-PGA degradation, enzymatic degradation was seen to be a milder technique that reduced the molecular weight of γ-PGA with better control and a narrower polydispersity, without disturbing the chemical constitution of the polymer (Yao *et al.*, 2009).

1.4.4 γ-PGA regulation

It was found that ComPA, DegSU and DegQ regulate γ-PGA production at the transcriptional level in response to quorum sensing, osmolarity and phase variation signals (Stanley and

Lazazzera, 2005). The ComPA system activates transcription of the *pgsBCA* operon at high cell density. DegSU activates transcription of *pgsBCA* in response to an increase in osmolarity. DegQ also regulates transcription of *pgsBCA* in an unknown fashion. SwrA regulates γ-PGA production post-transcriptionally, again, in an unknown manner (Stanley and Lazazzera, 2005).

1.5 CONFORMATION & ENANTIOMERIC STATES OF γ-PGA

1.5.1 Conformation of γ-PGA

Knowledge of the conformational state of γ-PGA is essential when it is used in medical/food applications since it is known that a small change in environmental conditions can change the properties of γ-PGA to a large extent. Depending on the environmental conditions, five different γ-PGA conformations – alpha helix, beta sheet, helix to random coil transition, random coil and enveloped aggregate – have been observed (Ho *et al.*, 2006a). The conformational state of γ-PGA can change depending on a number of factors. For instance, it has been shown that γ-PGA purified from *B. licheniformis* can exist in different conformational states depending on the concentration of γ-PGA and the pH of the solution (He *et al.*, 2000). At low concentration (0.1% w/v) and when the pH is below 7.0, γ-PGA adopts a conformation based largely on α-helices, whereas a β-sheet-based conformation predominates at higher pH. The β-sheet conformation seems to expose the negative charges of γ-PGA most efficiently. γ-PGA conformation is also sensitive to small changes in specific factors. For example, changes in PGA side-chain ionisation can have a pronounced effect on the conformation (Tiffany and Krimm, 1969; Shih and Van, 2001). A small amount of sodium chloride has also been shown to switch the preferred conformation of poly-L-glutamate from an extended conformation to a compact α-helix (Fedorov *et al.*, 2009).

1.5.2 Enantiomeric state of γ-PGA

In addition to conformation of γ-PGA, culture conditions also seem to have an impact on the enantiomeric composition of γ-PGA, which can alter its properties (Rehm, 2009). The enantiomeric composition of γ-PGA determines how γ-PGA is extracted after fermentation. If γ-PGA contains only L or D enantiomers, it is soluble in ethanol. However, if the enantiomers are mixed in equimolar amounts, then γ-PGA precipitates in ethanol (Candela and Fouet, 2006). As mentioned earlier (see **section 1.4.1**), the D and L isomer composition depend on a racemisation reaction, which is dependent on divalent cations (Leonard *et al.*, 1958). As discussed previously, Mn^{2+} affects the expression of the genes that code for enzymes responsible for racemisation reaction (Ashiuchi *et al.*, 2004). Other researchers have also confirmed that the enantiomeric composition of γ-PGA is dependent on Mn^{2+} in both *B. licheniformis* and *B. subtilis* (Wu *et al.*, 2006; Cromwick and Gross, 1995a). The ratio between L and D-glutamate in γ-PGA produced by *B. licheniformis* also depends on the concentration of Co^{2+} and Zn^{2+} (Cromwick and Gross, 1995a).

Optimization to determine the effect of the enantiomeric composition of γ-PGA on its behaviour and efficacy for a specific application is necessary. When γ-PGA with different enantiomeric composition was tested for its antifreeze properties, it was seen that the antifreeze activity of γ-PGA was not affected by its enantiomeric composition, but only by its molecular weight (Shih *et al.*, 2003). The antifreeze property was also seen to be affected by the concentration of cations in the order of $Mg^{2+} >> Ca^{2+} \sim Na^{+} >> K^{+}$. The effect of different enantiomeric composition of γ-PGA on other applications should also be identified.

1.6 PRODUCTION OF γ-PGA BY MICROBIAL FERMENTATION

Much research has gone into the production of γ-PGA from bacterial fermentation. γ-PGA producing bacteria usually produce and secrete the polymer into the medium in the late logarithmic and stationary phase of growth.

The most common genus used for producing γ-PGA is *Bacillus* and various strains of *B. licheniformis* and *B. subtilis* have been exploited for this purpose. It was Bovarnick in 1942 who first showed that γ-PGA is freely secreted into the medium after fermentation of *B. subtilis* (Bovarnick, 1942). After this, many researchers identified that *B. licheniformis* and *B. subtilis* secrete most of the product into the medium, which makes the recovery of the polymer relatively simple (Cheng *et al.*, 1989; Goto and Kunioka, 1992; Thorne *et al.*, 1954). The optimal nutrients that various bacteria can utilise to produce γ-PGA have been thoroughly investigated (Bajaj *et al.*, 2008; Cromwick *et al.*, 1996; Du *et al.*, 2005; Feng *et al.*, 2007; Huang *et al.*, 2011; Kazuki *et al.*, 2007; Kedia *et al.*, 2010; Ko and Gross, 1998; Wu *et al.*, 2010b; Xu *et al.*, 2005a; Yamashiro *et al.*, 2011).

Bacteria can produce γ-PGA with different properties, such as molecular weight and enantiomeric composition (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007; Shih and Van, 2001; Sung *et al.*, 2005). γ-PGA can also be produced in its pure acid form or as a salt of the polymer (**Fig 1.8**) and it can be crystalline or amorphous (Ho *et al.*, 2006a). These properties can determine the application for which γ-PGA is used. The medium composition is one of the factors that can influence the properties of γ-PGA and can be used to control them (Bajaj and Singhal, 2011; Shih and Van, 2001).

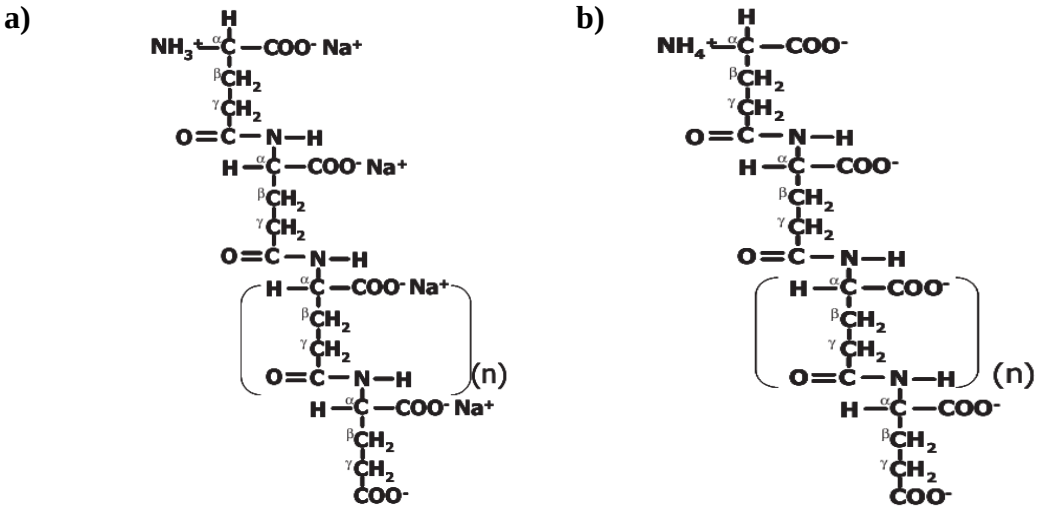


Fig 1.8: Structure of a) Na salt of γ-PGA b) Acid form of γ-PGA (Sung *et al.*, 2005).

Hence, the medium used to produce γ-PGA by various bacteria is crucial because it directly affects the properties of γ-PGA. The nutrient requirements and growth conditions vary with the bacterial strain used for production. Some of the factors affecting γ-PGA production in different bacteria have been enumerated in the following sections.

1.6.1 Effect of carbon and nitrogen source on γ-PGA production

Choosing the appropriate C source that can be utilized by bacteria can help produce γ-PGA efficiently. An interesting study has been done to understand the effect of different carbon sources (glucose and glycerol) on the yield, cell growth and molecular weight of γ-PGA produced from *B. licheniformis* (Ko and Gross, 1998). It was seen that glucose was consumed much quicker and the cell growth was better with glucose as the carbon source. However, the yield increased considerably (from 5.7 to 20.5 g/L) when a combination of glycerol and glucose was used rather than glucose on its own, although glucose was still consumed much quicker than glycerol. The actual mechanism by which glycerol causes an increase in the yield of γ-PGA was a mystery until the work done by Du and co-workers (2005), where the

effect of glycerol on γ-PGA production with a mutant strain of *B. licheniformis* ATCC 9945A (also called *B. licheniformis* WBL-3) was studied (Du *et al.*, 2005). It was seen that the yield of γ-PGA increased from 9.7 to 16.7 g/l as the concentration of glycerol was increased in the medium from 0 to 70 g/l. Above this concentration the yield of γ-PGA started decreasing. The reason for the increase in yield was found to be due to a change in the phospholipid composition of the cell membrane due to the addition of glycerol, which increased the permeability of the cell membrane for γ-PGA. Without the use of glycerol (only citric acid and glutamic acid were used in this case), the intracellular concentration of γ-PGA was quite high which hampered the synthesis of γ-PGA. The importance of glycerol was highlighted in another study, where it was seen that addition of glycerol during production of γ-PGA using *B. subtilis* NX-2 was seen to reduce broth viscosity which resulted in an increase in the uptake of other nutrients, which in turn resulted in a higher yield and reduction in molecular weight of γ-PGA (Wu *et al.*, 2010a). *B. subtilis* NX-2 was earlier isolated from soil by Xu *et al* and γ-PGA production was optimized recently to produce 42 g/l of γ-PGA in a fed batch culture (Xu *et al.*, 2005a; Yao *et al.*, 2010).

Recently, Yamashiro and co-workers (2011) investigated the effect of different carbon sources on the production of γ-PGA using *B. subtilis* chungkookjang (Yamashiro *et al.*, 2011). They found that D-glucose and D-fructose were effective for γ-PGA production. However, D-galactose and lactose were not as effective. It was also found that pentoses such as xylose and arabinose can be utilized effectively for γ-PGA production. This finding is useful, as these pentoses are derived from inedible biomasses which could lead to reduction in raw material cost for mass production of γ-PGA. In addition, the induction of the *pgsE*-gene product using Zn^{2+} as an activator increased the productivity of γ-PGA three fold (Yamashiro *et al.*, 2011).

For *B. subtilis* ZJU-7, it was found that glucose was a better C source for cell growth, however, more γ-PGA was produced when sucrose was used in the medium (Shi *et al.*, 2006; Xu *et al.*, 2005a). Among the nitrogen sources, tryptone was most effective, both in terms of cell growth and γ-PGA production. The optimized medium gave a maximum yield of 58.2 g/l after 24h of cultivation. This is the highest yield of γ-PGA obtained thus far using *B. subtilis* in submerged fermentation. Chen *et al.* (2010) demonstrated that replacing tryptone with yeast extract paste can make the large scale production of γ-PGA (100 l fermenter) from *B. subtilis* ZJU-7 cost-effective by reducing the cost of nitrogen source needed to produce 1 kg of γ-PGA from $161.60 (tryptone) to $1.80 (yeast extract paste) (Chen *et al.*, 2010). The highest yield (54 g/l) achieved using yeast extract as the nitrogen source was only slightly lower than the yield of γ-PGA produced when tryptone was used as the nitrogen source (58.2 g/l) (Xu *et al.*, 2005a).

Kedia and co-workers (2010) also investigated the effect of different media on the production of γ-PGA in *B. subtilis* natto (Kedia *et al.*, 2010). Of the media tested (Medium E, C, F and GS), GS medium, with sucrose as C source, was most effective for γ-PGA production, resulting in the production of 26-28 g/l of γ-PGA after 96 h. When medium C with glucose as C source was used to produce γ-PGA, the yields obtained were lower than when GS medium with sucrose was used. In fact, when glucose at 80 g/l was used in medium C, the precipitated γ-PGA seemed to contain impurities (non-consumed glucose, yeast extract and peptone).

As has been discussed previously (see **section 1.4.2**), bacteria utilize exogenously provided L-glutamic acid for the production of γ-PGA. However, in some instances glutamic acid independent γ-PGA production has been shown (Ito *et al.*, 1996). *B. subtilis* TAM-4 produced γ-PGA in the presence of ammonium salt and different sugars. On evaluating the

effect of C sources on γ-PGA production, it was found that fructose was the best C source. Likewise, it was found that ammonium chloride was the best nitrogen source for polymer production with this bacterial strain. *B. subtilis* TAM-4 produced 22.1 g/l of γ-PGA without any polysaccharide by-products when grown on 1.8% ammonium chloride and 7.5% fructose at 30˚C for 96h (Ito *et al.*, 1996).

1.6.2 Effect of salts on γ-PGA production

Salts in the medium can also affect properties of the produced γ-PGA, such as molecular weight. For instance, when NaCl concentration in the medium was increased from 0% to 4%, the molecular weight of the resultant γ-PGA produced by *B. licheniformis* 9945a increased from 1.2 x 10^6 Da to 2.2 x 10^6 Da (Birrer *et al.*, 1994). A higher salt concentration in the medium used to grow *B. megaterium* also produced γ-PGA with a higher molecular weight (Kazuki *et al.*, 2007). 5% NaCl produced γ-PGA with a molecular weight of 1 x 10^6 Da, whereas 10% NaCl increased the molecular weight to 2 x 10^6 Da. The salt concentration also seemed to affect the enantiomeric composition of γ-PGA. In another study, γ-PGA produced by *B. subtilis* chungkookjang was affected differently by salt concentration in production medium (Sung *et al.*, 2005). Interestingly, a high-molecular weight polymer (>2000 KDa) was synthesized at low NaCl concentrations (0.5%) whereas a lower molecular weight polymer (10-200 KDa) was produced when the NaCl concentration was increased (>10%). This is in contrast to the study by Birrer and co-workers (1994) with *B. licheniformis*, where increasing the salt concentration increased the molecular weight (Birrer *et al.*, 1994), thus indicating that molecular weight is dependent on both bacteria and medium used for γ-PGA production.

Ammonium salts could also affect γ-PGA production and cell growth. More specifically, ammonium sulphate was seen to be useful for decreasing cell growth, increasing γ-PGA productivity and producing a high molecular weight polymer from *B. subtilis* IFO3335 without by-product formation (Feng *et al.*, 2007; Goto and Kunioka, 1992; Kaplan, 1998). When the ammonium sulphate concentration was increased from 0 to 10 g/l, an increase in γ-PGA yield from 0.8 to 10 g/l was observed, without any polysaccharide by-product formation (Kaplan, 1998). In fact, a high concentration of ammonium sulphate also caused the γ-PGA produced by *B. subtilis* subsp. chungkookjang to be richer in L-glutamate than its D-enantiomer (Park *et al.*, 2005).

Recently, the effect of $CaCl_2$ was tested on the production of γ-PGA in *B. subtilis* CGMCC 2108 (Huang *et al.*, 2011). Addition of $CaCl_2$ reduced broth viscosity and increased consumption of extracellular glutamate by 11.4%. It also increased γ-PGA yield from 7.88 g/l to 9.07 g/l. It was found that $CaCl_2$ increased activities of the three enzymes involved in γ-PGA synthesis at the 2-oxoglutarate branch of the TCA cycle (isocitrate dehydrogenase, glutamate dehydrogenase and 2-oxoglutarate dehydrogenase complex). Addition of $CaCl_2$ seemed to ensure that more oxoglutarate was converted into glutamate which could then be incorporated into the growing γ-PGA chain. The molecular weight remained unaffected with changes in $CaCl_2$ concentration. This is the first report where the effect of Ca^{2+} has been shown to affect γ-PGA production.

1.6.3 Effect of pH on γ-PGA production

The optimum pH of the medium is crucial for γ-PGA production and differs for different bacteria. It has been shown that pH 6.5 is optimal for γ-PGA produced by *B. licheniformis* 9945a, since citric acid was utilized better at this pH when compared to other pH values

(Cromwick *et al.*, 1996). In another study (Richard and Margaritis, 2003b), optimizing the pH of *B. subtilis* IFO3335 fermentation to pH 7 increased the yield of γ-PGA to 23 g/l, which was 1.44 times greater than that observed in a different study (Kunioka and Goto, 1994) with the same bacteria, where pH was not optimized.

In some cases, a two stage pH shift strategy needs to be employed when the optimal pH for growth and γ-PGA production are different. For instance, when γ-PGA production in *B. subtilis* CGMCC 0833 was analysed, it was found that the optimum pH for glutamate utilization was 6.5 (Wu *et al.*, 2010b). However, the optimum pH for cell growth was found to be pH 7.0. Hence, a two stage pH shift strategy was proposed, where for the first 24 h the pH was kept at pH 7.0 for maximum cell growth, following which the pH was lowered to pH 6.5 for maximum utilization of glutamate. Using the pH shift strategy, glutamate utilization was increase from 24.3 to 29.5 g/l and the yield of γ-PGA was increased by 24.8%.

1.6.4 Effect of metabolic precursors on γ-PGA production

An increase in the yield of γ-PGA has been achieved by addition of small quantities of precursors for γ-PGA production (Bajaj and Singhal, 2009a). When *B. licheniformis* NCIM 2324 was fed with L-glutamine (0.07 g/l) and α-ketoglutaric acid (1.46 g/l) in addition to the basal medium, the γ-PGA yield increased to 35.75 g/l, in contrast to 26.12 g/l when no metabolic precursors were added. The added precursors also helped improve consumption of L-glutamic acid. In another study using *B. subtilis* R23, only α-ketoglutaric acid was seen to improve γ-PGA yield (Bajaj and Singhal, 2009b) while L-glutamine had no effect on polymer yield. Sequential optimization technique caused the yield of γ-PGA to increase from 7.64 to 25.38 g/l. These studies demonstrate that addition of relatively inexpensive metabolic

precursors can lead to a reduced cost in γ-PGA production, since small quantities are converted into a high-value multifunctional biopolymer.

1.6.5 Effect of aeration/agitation on γ-PGA production

γ-PGA production makes the medium viscous, which hampers the mass transfer rate of oxygen and nutrients. This makes control and maintenance of aeration and agitation extremely crucial for γ-PGA production. When Cromwick *et al.* (1996) increased the aeration from 0.5 l/min to 2.0 l/min and agitation from 250 rpm to 800 rpm during production of γ-PGA with *B. licheniformis*, the yield increased significantly from 15 g/l to 23 g/l (Cromwick *et al.*, 1996). Other effects included rapid consumption of L-glutamic acid and citric acid and a lower molecular weight product. Even though the agitation and aeration was increased, the partial pressure of oxygen (pO_2) still decreased (to < 1%) by 29 hours. The lower transfer rate of oxygen could be due to two reasons. It could either be due to increased viscosity of the culture media on γ-PGA production, or because the oxygen demands of the high cell density culture could not be sustained by the system.

1.6.6 Effect of Mn^{2+} on γ-PGA production

As mentioned previously (see **section 1.4.1 & 1.5.2**), change in concentration of Mn^{2+} can have several effects on the production of γ-PGA. Recently, Kedia and co-workers (2010) demonstrated the importance of $MnSO_4$ during the growth of *B. licheniformis* 9945a and production of γ-PGA (Kedia *et al.*, 2010). When $MnSO_4$ was absent in the medium, cell viability started to decline after 50 h of incubation. Medium containing 2.46 mM $MnSO_4$ showed no loss in viability after 96 h. Presence of $MnSO_4$ also caused the cells to assimilate glycerol, L-glutamic acid and citric acid better as compared to when $MnSO_4$ was absent. In

addition, presence of $MnSO_4$ in the medium increased the yield of γ-PGA (13 g/l) as compared to when no $MnSO_4$ was used (5 g/l). Hence, $MnSO_4$ can be used appropriately to achieve prolonged cell viability, better utilization of nutrients and increased yield of γ-PGA production.

Until the work done by Cromwick and Gross (1995a), which finally resolved the debate, there was divided opinion regarding Mn^{2+} affecting the enantiomeric composition of γ-PGA (Cromwick and Gross, 1995a). This study showed that the proportion of L-glutamic acid in γ-PGA varies inversely with $MnSO_4$ content. When the concentration of $MnSO_4$ was increased from zero to 615 μM, the L-glutamic acid repeating units in γ-PGA decreased from 50% to 10% (Cromwick and Gross, 1995a). Ca^{2+} did not seem to affect the percentage of D-glutamic acid. The effect of Mn^{2+} on γ-PGA was confirmed by another study (Perez-Camero *et al.*, 1999) where the concentration of Mn^{2+} was found to affect the enantiomeric composition of γ-PGA, especially when it was present in low concentrations (≤ 20 μM) in the medium. Varying Mn^{2+} from 0 to 1230 μM resulted in polymers ranging between 10 to 90% D-units and with molecular weights between 4×10^5 to 2.0×10^6 Da. It has been shown that Mn^{2+} affected the stereochemical and enantiomeric composition of γ-PGA produced with *B. subtilis* NX-2 as well (Wu *et al.*, 2006). When the concentration of Mn^{2+} varied from 0 to 0.09 g/L, the proportion of D-glutamate increased from 18 to 77%. Mn^{2+} seemed to affect the stereochemical properties of γ-PGA by altering the activity of glutamate racemase (Wu *et al.*, 2006). Controlling the enantiomeric composition of γ-PGA could be useful since γ-PGA with different D/L compositions could be used for different applications.

1.6.7 Effect of cell wall lytic enzymes on γ-PGA production

Recently, Mitsui and co-workers (2011) were first to report the role of cell wall lytic enzymes in γ-PGA production (Mitsui *et al.*, 2011). More specifically, it was seen that Cwl0, a cell wall lytic enzyme belonging to a D,L-endopeptidase was found to play an important role in γ-PGA production and also affected the molecular size of the polymer. Cwl0 was found to be the major enzyme responsible for degradation of γ-PGA in *B. subtilis* natto (Mitsui *et al.*, 2011). Disruption of the *cwl0* gene increased the yield of γ-PGA produced while producing a higher molecular weight polymer.

Some interesting examples of γ-PGA production are summarized in **Table 1.1** below.

Table 1.1: Examples of γ-PGA production in different organisms

Organism	Comments	References
B. subtilis chungkookjang	Produces super-high-molecular-weight γ-PGA - 2 x 10^6 Da without the formation of any by products. Promising for various industrial applications.	(Park *et al.*, 2005)
B. subtilis CCTCC202048	γ-PGA produced using solid state fermentations. Maximum γ-PGA production of 83.61 g $kgds^{-1}$ was obtained.	(Jian *et al.*, 2005)
B. licheniformis NCIM 2324	γ-PGA produced using solid state fermentations. Maximum yield of 98.64 g $kgds^{-1}$ was obtained.	(Bajaj *et al.*, 2008)
B. licheniformis strain-R	γ-PGA production by cells immobilized using an agar-alginate gel beads mixture (γ-PGA yield – 36.75 g/l). Luffa pulp-adsorbed cells produced γ-PGA yield of 50.4 g/l. First study that uses immobilized cells for γ-PGA production.	(Berekaa *et al.*, 2009)
B. licheniformis S2	Newly isolated strain shown to have ability to reduce ammonium nitrogen content in swine manure by converting ammonium into biomass and γ-PGA. Bacteria could be used to prevent eutrophication of ground and surface water and pollution of atmosphere.	(Hoppensack *et al.*, 2003)
Bacillus sp. RKY3	Newly isolated strain used for γ-PGA production. Maximum yield of 48.7 g/l was achieved using optimized medium in a 3 l volume lab-scale fermenter.	(Jeong *et al.*, 2010; Jung *et al.*, 2005)
B. subtilis NX-2 & *Corynebacterium glutamicum*	*Corynebacterium glutamicum* have the ability to produce high levels of glutamic acid. Co-culturing bacteria for γ-PGA production eliminated the need to add exogenous L-glutamic acid. Reduced fermentation time and production cost.	(Xu *et al.*, 2005b)
B. mesentericus MJMI	Bacteria isolated from Korean domestic bean paste. γ-PGA consisted of 2000 glutamic acid residues.	(Zhao *et al.*, 2005)
E. coli	*pgsBCA* genes that are responsible for γ-PGA production were cloned and expressed in *E. coli*. Low yields of γ-PGA were obtained (~0.024 g/l)	(Ashiuchi *et al.*, 1999)
Tobacco leaves	γ-PGA production in tobacco leaves was achieved by introducing the pgsBCA complex via *Agrobacterium* infection. 600 µg of γ-PGA per gram of leaf material was obtained.	(Tarui *et al.*, 2005)

It is evident that the appropriate carbon and nitrogen source for γ-PGA production would depend on the bacteria used for its production. Some bacteria utilize different carbon sources for growth and γ-PGA production at different pH. In addition, cost of production could be reduced by replacing an expensive nutrient with an inexpensive one which does not change the productivity significantly, hence optimization of these nutrients is important before industrial production of γ-PGA.

1.7 APPLICATIONS OF γ-PGA

γ-PGA is an extremely important substance that has been exploited for a wide array of useful applications due to its unique properties. It is biodegradable, edible and non-toxic for humans, which are prerequisites for the polymer to be used for human benefit. Moreover, γ-PGA breaks down into its component glutamic acid residues in the body. These can enter normal cellular metabolism and the polymer is not excreted by the kidney (Singer, 2005).

1.7.1 Medical applications of γ-PGA

Previously, α-PGA has been used for medical applications (Hashida *et al.*, 1999; Li *et al.*, 1998; Zou *et al.*, 2007). However, multiple researchers have shown that using γ-PGA for medical applications could be more promising, because it cannot be broken down in the body by α-proteases (Hsu and Lin, 2007; Shih and Van, 2001; Ye *et al.*, 2006). Therefore, medical applications of γ-PGA have attracted a lot of interest in the last decade and much research has been carried out in this area. Some of the more important potential medical applications of γ-PGA are summarized in **Table 1.2**.

Table 1.2: Potential applications of γ-PGA and its derivatives

Potential Application	Findings/Benefit	References
Drug carrier	Yield of cis-dichlorodiammineplatinum*(II)* (CDDP) incorporation into γ-PGA was 12.3% better than α-PGA. Higher antitumour activity than CDDP alone, while being less toxic than the free drug. Can potentially be used for treatment of breast cancer.	(Ye *et al.*, 2006)
	Improved sustained delivery of diphenhydramine (DPH) when administered as a complex with γ-PGA. Complex was less stable in weak acidic or neutral pH and more stable in high acidic pH. γ-PGA was also useful for blocking the bitter taste of DPH.	(Agresti *et al.*, 2008)
Protein carrier	Sustained delivery of bone morphogenetic proteins for bone regenerative therapy.	(Hsieh *et al.*, 2006)
	Potential vaccine carrier for systemic and mucosal administration. No cytotoxic effect.	(Akagi *et al.*, 2005)
	Effective oral delivery of insulin. Intestinal adsorption of insulin was enhanced & prolonged reduction in blood glucose level was achieved.	(Sonaje *et al.*, 2010)
Monoclonal antibodies	No deactivation and increased stability of monoclonal antibodies when administered in γ-PGA nanoparticles.	(Sung *et al.*, 2009)
Gene delivery	γ-PGA complexes were used for effective gene delivery with low toxicity along with high uptake and gene expression.	(Kurosaki *et al.*, 2009; Kurosaki *et al.*, 2010)
Antitumourogenic agent	Oral administration of γ-PGA induced Natural Killer (NK) cell-mediated antitumour immunity in mice bearing MHC class I-deficient tumours.	(Kim *et al.*, 2007)
Antimutagenic food additive	γ-PGA suppressed SOS response of *Salmonella typhimurium.*	(Sato *et al.*, 2008)
Biodegradable scaffold	γ-PGA fibres cross-linked with cystamine improved growth of mouse L929 fibroblast cells. Can be used for biomedical and tissue engineering applications.	(Yoshida *et al.*, 2009)
	Better growth of rat osteosarcoma cells on γ-PGA chitosan matrices than on chitosan alone.	(Hsieh *et al.*, 2005)
Biological antiadhesive	Cross-linked γ-PGA reduced tissue adhesion over injured surfaces in a rat model by forming a viscous hydrogel over it.	(Izumi *et al.*, 2007)
Soft tissue adhesive	γ-PGA/gelatin hydrogels used as tissue adhesive. The hydrogel was more stable than fibrin glue.	(Hsu and Lin, 2007)
Wound healing	γ-PGA complexes were shown to have good wound healing properties.	(Lee *et al.*, 2011; Tsao *et al.*, 2010)
Antibacterial activity	γ-PGA-coated magnetite nanoparticles exhibited antibacterial activity against *Salmonella enteritidis* SE01.	(Inbaraj *et al.*, 2011)
Calcium absorption	Administration of γ-PGA increased calcium absorption in the intestine in post-menopausal women by inhibition of formation of an insoluble calcium complex with phosphate. Can be potentially used for treatment of bone disorders.	(Tanimoto *et al.*, 2007)
Inhibition of influenza virus	γ-PGA based glycopolymers (used to inhibit influenza virus) showed higher solubility in water & heat stability and lower toxicity & immunogenicity compared to glycopolymers without γ-PGA.	(Ogata *et al.*, 2009)
Glucose sensor	Needle-type glucose sensors prepared by covalent immobilization of glucose oxidase on γ-PGA film. Increased stability of the electrode to a month.	(Yasuzawa *et al.*, 2011)
Treatment of xerostomi (dry mouth)	γ-PGA promoted salivary secretion and produces moisturizing effect. Solved problems associated with sticky displeasure of the mouth, difficulties in speaking, bad breath, dental caries, periodontal disease and mucosal infectious disease.	(Uotani *et al.*, 2011)
Nutrition supplement	γ-PGA may increase *in vivo* bioavailability and solubility of calcium & magnesium, reduced progress of osteoporosis, facilitated growth of osteoblast cells, reduced calcium loss and maintained bone strength.	(Ho *et al.*, 2006b)

γ-PGA remains stable in a low pH environment by conforming to an α-helical structure and disintegrates in a weaker acidic or neutral environment. This property of γ-PGA has been exploited for sustainable delivery of various drugs and proteins that are often destroyed or degraded quickly when administered orally, due to the harsh conditions of the gastrointestinal tract.

For instance, Agresti *et al*. (2008) have been successful in making a complex of Diphenhydramine (DPH) and α-helical γ-PGA (Agresti *et al.*, 2008). DPH is a first generation antihistamine drug and has a bitter taste. The use of this complex was found to be advantageous for two reasons. Firstly, the DPH/γ-PGA complex was less stable at neutral and weak acidic pH (5-7) and hence, it dissolved readily and completely. At neutral pH (7.2) more than 95% of DPH was released from the complex within 20 minutes. The α-helical structure collapse of γ-PGA was thought to be responsible for the pH-induced dissolution of the complex. The complex was seen to be stable in a pH range of 2.0 - 4.5, with partial dissolution observed at pH 4.5. Secondly, γ-PGA was effective in blocking the bitter taste of DPH.

The oral administration of insulin is beneficial but challenging, due its instability in the gastrointestinal tract. Recently, nanoparticles comprising chitosan and γ-PGA have been used for effective oral delivery of insulin (Sonaje *et al.*, 2010). The nanoparticles were freeze dried and filled inside an enteric-coated capsule. Freeze drying did not seem to affect the internal structure and pH stability of the nanoparticles. The insulin release was shown to be pH dependent. Intestinal absorption of insulin was enhanced and a prolonged reduction in blood glucose level was achieved. This formulation could potentially be used for oral delivery of other therapeutic proteins as well.

γ-PGA has also been used to develop a non-viral vector for safe gene delivery, where a complex of γ-PGA, pDNA and polyethylenimine (PEI) complex was used (Kurosaki *et al.*, 2009). A pDNA/PEI complex, when used alone, shows high transgene efficiency, but is accompanied with agglutination with erythrocytes and extremely high cytotoxicity. Addition of γ-PGA to this complex contributed to low toxicity and agglutination, along with exhibiting high uptake and gene expression. In the follow up study, safe and effective gene delivery vectors using γ-PGA to coat polyplexes and lipoplexes were developed (Kurosaki *et al.*, 2010). γ-PGA coated vectors showed high transfection efficiencies with markedly low toxicities.

γ-PGA, on its own, has been shown to have potential antitumourogenic activity and could play a role in cancer immunotherapy (Kim *et al.*, 2007). Oral administration of γ-PGA, isolated from *B. subtilis* chungkookjang, with a molecular weight as high as 2 x 10^6 Da, induced significant Natural Killer (NK) cell-mediated antitumour immunity in mice bearing MHC class I-deficient tumours. γ-PGA with lower molecular weight (10 and 100 kDa) did not generate significant antitumour activity which suggests that molecular weight is a critical factor in optimizing the antitumour applications of γ-PGA. The mechanism underlying the antitumour immunity was thought to be due to the activation of NK cells by γ-PGA rather than a direct cytotoxic effect.

γ-PGA could also be used to make a novel biocompatible and biodegradable scaffold for biomedical and tissue engineering applications (Yoshida *et al.*, 2009). Among others, it has also been shown to be beneficial as a biological adhesive for sutures (Hsu and Lin, 2007; Shih and Van, 2001), as an antiadhesive (Izumi *et al.*, 2007), as a wound healing agent (Lee *et al.*, 2011; Tsao *et al.*, 2011), as an antibacterial agent (Inbaraj *et al.*, 2011; Tsao *et al.*,

2010), for improvement of calcium absorption in the intestine (Tanimoto *et al.*, 2007; Sung *et al.*, 2005), as a component in an agent for inhibition of influenza virus (Ogata *et al.*, 2009) and for increased stability of a glucose sensor (Yasuzawa *et al.*, 2011), for treatment of xerostomia (Uotani *et al.*, 2011) and as a nutritional supplement (Ho *et al.*, 2006b).

1.7.2 Food applications of γ-PGA

γ-PGA is food derived, since it is naturally present in the mucilage of natto (fermented soybeans) and chungkookjang, which are traditional foods in Japan and Korea respectively. In addition, γ-PGA is generally recognized as safe (GRAS) by the FDA (Shyu and Sung, 2010) and hence has been utilized for different food applications.

One of the prominent applications of γ-PGA in food is as an antifreeze agent. Preservation of foodstuffs by freezing is an effective and popular method. However, when it is frozen and thawed, there are undesirable effects, such as deterioration of living cells and foods due formation of ice crystals. These deleterious effects can be prevented by the addition of antifreeze agents, which exhibit antifreeze activity by reducing the freezing point of the product they are added to. It has been found that certain salts of acidic amino acids (mainly sodium salts) show good antifreeze properties (Shih and Van, 2001). Glucose is considered to be an agent with excellent antifreeze activity (Mizuno *et al.*, 1997). Mitsuiki *et al.* (1998) investigated the antifreeze activities of γ-PGA using differential scanning calorimetry and found that γ-PGA, which had a molecular weight less than 20,000 Da, showed an antifreeze activity much higher than that of glucose (Mitsuiki *et al.*, 1998). The antifreeze activity of γ-PGA produced from *B. licheniformis* CCRC 12826 was also analysed in another study (Shih *et al.*, 2003). Different molecular weights and enantiomeric compositions and salts were tested. Lower molecular weight γ-PGA was shown to have a higher antifreeze activity. The

antifreeze activity was unaffected by enantiomeric composition. It was also seen to be dependent on cations decreasing in the following order – Mg^{2+} >> Ca^{2+} ~ Na^{+} >> K^{+}. There are two distinct advantages when γ-PGA is used as an antifreeze agent. Firstly, it has a weak taste when compared to the conventional agents used, such as saccharides, inorganic salts and amino acids. This means the taste and palatability of food would not change, even if γ-PGA is added in large quantities. Secondly, when γ-PGA is used with high mineral food preparations, it accelerates the absorption of minerals in the small intestine (Ashiuchi, 2010). In addition, it is also successful in masking the taste of the enriched minerals (Ashiuchi, 2010).

γ-PGA has also been used as a bitterness relieving agent (Sonoda *et al.*, 2000), an agent for prolonging shelf life in bakery products and noodles (Kunno *et al.*, 1988a; Kunno *et al.*, 1988b), a texture enhancing agent, an ice-cream stabilizer and a thickener for fruit juice beverages (Yamanaka and Kikuchi, 1991). The use of γ-PGA has been shown to significantly improve the rheological and thermal properties of wheat dough (Soliman *et al.*, 2005). γ-PGA addition made wheat bread softer and eventually retarded staling of bread. More recently, it has been used to improve the emulsion stability of sponge cake. A high molecular weight Na salt of γ-PGA (> 10^6 Da) delayed the staling of cake crumb as well as improved the texture of the cake (Shyu and Sung, 2010).

1.7.3 Other applications of γ-PGA

Due to its biodegradability, heavy metal binding capability and high flocculating activity, γ-PGA can be potentially used in wastewater treatment plants, in the drinking water processing industry and in downstream processing for food and fermentation industries (Shih and Van, 2001). Recently, due to its high flocculating activity, γ-PGA was shown to be effective in treatment of vinnase to remove suspended solids (Octavio *et al.*, 2012). Vinnase is the waste

water produced from ethanol distillation and is difficult to dispose of. One of the reasons why this is difficult is due to its low pH. The low pH of vinnase actually enhanced γ-PGA flocculating efficiency. Also, pre-treatment processes were not required, making γ-PGA ideal for disposal of vinnase in combination with a mild chemical treatment for removal of coloured compounds (Octavio *et al.*, 2012).

The preparation and characterisation of novel biodegradable nanoparticles based on complexation of γ-PGA (derived from *B. licheniformis* 9945a) with bivalent lead ion (Bodnár *et al.*, 2008) has also been investigated. The prepared complex was stable in aqueous media at low, neutral and mild alkaline conditions. This complex is thought to have useful water treatment applications due to its biodegradability along with heavy metal binding activity.

γ-PGA (molecular weight: 9.9 x 10^5 Da) has been used effectively to remove basic dyes from aqueous solution (Inbaraj *et al.*, 2006). It was found that 98% of the dye adsorbed on γ-PGA could be recovered at pH 1 which facilitates the reuse of spent γ-PGA. As γ-PGA is non-toxic and biodegradable, the adsorption system developed could offer green solutions to the dye industry. More recently, magnetite particles were coated with γ-PGA and were used for their methylene blue dye adsorption capability. The particles were stabilized in deionized, tap and river waters as well as in acidic and basic media. The dye was completely recovered using these particles by lowering the pH to 1.0. These magnetite particles coated with γ-PGA could be used for removal of cationic dyes from wastewaters (Inbaraj and Chen, 2011).

γ-PGA has successfully been used as an active ingredient in a hyaluronidase inhibitor, demonstrating its utility in the cosmetic industry (Sung *et al.*, 2007). Hyaluronidase is an enzyme that degrades hyaluronic acid present in the skin dermis. The composition was tested

on fifty women (age group between 30 and 50) and was shown to maintain skin elasticity by inhibiting the activity of hyaluronidase and reducing allergenic reactions by inhibiting the permeability of inflammatory cells (Sung *et al.*, 2007).

Other interesting applications of γ-PGA include using it as a component in biodegradable golf balls (Egashira and Takehana, 2011) and as a protein crystallization inducer (Hu *et al.*, 2008).

1.7.4 Optimization of γ-PGA for applications

As previously discussed, since γ-PGA with different properties is useful for different applications, optimization of γ-PGA with respect to its properties is very important. Properties such as molecular weight, conformational state, enantiomeric composition and form of the polymer can affect the efficiency of γ-PGA for a particular application. Probably the most decisive property of γ-PGA is its molecular weight. For example, polymers of different molecular weights are required to control a drug's release into the tissue. In this effort (Richard and Margaritis, 2006), it was found that the molecular weight of γ-PGA produced by bacteria is usually higher than that required for drug delivery applications. The molecular weight of PGA required for conjugation with Paclitaxel, for example, has been determined to be 3×10^4 - 6×10^4 Da (Li *et al.*, 1998). The molecular weight of γ-PGA required would differ depending on the drug and application. Molecular weight reduction can be an important step in producing PGA for a drug delivery application. Different techniques used for this purpose are ultrasonic degradation, alkaline hydrolysis, alteration of medium composition and microbial or enzymatic degradation (Shih and Van, 2001).

Ultrasonic degradation in particular was shown to be an effective method to reduce both the molecular weight and the polydispersity of naturally produced PGA, without disturbing the chemical structure of the polymer (Graciela *et al.*, 2000). In another study, *in situ* depolymerisation of γ-PGA in the cell-free fermentation broth of *B. subtilis* IFO 3335 was carried out (Richard and Margaritis, 2006). The molecular weight, when measured with the help of Gel Permeation Chromatography (GPC) and intrinsic viscosity correlations, reduced from about 4. x 10^6 Da to 5.5 x 10^4 Da over a period of 144 h. As with the study done by Yao *et al.*, the polydispersity of γ-PGA decreased as a function of hydrolysis time (Yao *et al.*, 2009). Enzymatic degradation seems to be a better method to obtain γ-PGA of the required molecular weight in a controlled fashion. Simple hydrolysis of γ-PGA in aqueous solution has also been studied at different temperatures (80˚C, 100˚C and 120˚C) (Goto and Kunioka, 1992). γ-PGA was rapidly hydrolysed from 226 kDa to 18 kDa in 30 mins at 120˚C. Hydrolysis was much slower at lower temperatures. Hydrolytic degradation of γ-PGA was found to proceed through random chain scission.

1.8 SUMMARY

γ-PGA is biodegradable, edible and non-immunogenic. Because of this it can be safely used for a variety of applications, which are fast increasing. Knowledge of the genes and enzymes involved in γ-PGA regulation throws light on the conditions that support γ-PGA production (for e.g. high osmolarity). This information could be used to isolate and screen new γ-PGA producing strains from different sources. Since every application of γ-PGA exploits specific properties of γ-PGA, it would be interesting to compare different γ-PGA producing bacteria and analyse the properties of the produced γ-PGA in these organisms.

For conventional materials to be replaced with new sources of γ-PGA, the cost of production of γ-PGA needs to be several tens to hundreds of times lower than what it is at present. Using cheap nutrients for production of γ-PGA is one way to reduce costs. For instance, corn steep liquor, which is an agricultural waste product, could be used as an effective nitrogen source for γ-PGA production (Jung *et al.*, 2005). In addition, a complete understanding of γ-PGA synthesis, especially regulation of γ-PGA production within the cell, would help in making the production process more economical. Statistical analyses of large scale γ-PGA production could also lead to answers for cost-effective production of γ-PGA. Bajaj and Singhal have shown that sequential optimization approach using statistical analysis (Plackett-Burman design and response surface methodology) is a useful way of optimizing the nutrients required for γ-PGA production (Bajaj and Singhal, 2009b; Bajaj *et al.*, 2008; Bajaj *et al.*, 2009; Bajaj and Singhal, 2009a). Addition of cheap γ-PGA precursors in small quantities that can increase the yield of γ-PGA produced could also make the production process more cost-efficient.

α-PGA has been widely exploited for medical applications, chiefly for sustained drug delivery. However, researchers have pointed out that there could be several advantages of using γ-PGA over α-PGA (Hsu and Lin, 2007; Shih and Van, 2001; Ye *et al.*, 2006). Pure γ-PGA can be obtained in large quantities without any chemical modification steps. It is not susceptible to proteases and hence could provide better sustained delivery of conjugated drugs in the body. Better incorporation of drug in terms of quantity of drug conjugated into γ-PGA has been achieved.

γ-PGA can exhibit different properties and it is important to choose γ-PGA with appropriate properties for a specific application. Optimization of γ-PGA with respect to molecular

weight, enantiomeric composition and conformational states is an indispensable step in making specific applications of γ-PGA practical. The *Bacillus* genus has thus far dominated the production of γ-PGA. Various strains of *Bacillus* have been researched for γ-PGA production (Bajaj *et al.*, 2009; Birrer *et al.*, 1994; Du *et al.*, 2005; Bovarnick, 1942; Jian *et al.*, 2005; Kubota *et al.*, 1993; Park *et al.*, 2005; Zhao *et al.*, 2005; Liu *et al.*, 2011). It is essential to investigate the production of γ-PGA in bacteria that have not previously been used for this purpose. These bacteria could produce γ-PGA with higher yields or with properties suitable for particular applications. The aim of this work was to investigate and contribute the production of γ-PGA in eight different strains of *Bacillus*. Six of these bacteria have not been previously used for production of this multifunctional biopolymer. It is known from previous research that *B. subtilis* and *B. licheniformis* are able to produce extracellular γ-PGA which can be recovered from the production medium (Bajaj *et al.*, 2008; Bajaj *et al.*, 2009; Birrer *et al.*, 1994; Buescher and Margaritis, 2007; Shih and Van, 2001; Xu *et al.*, 2005a). In this work, five strains of *B. subtilis* - *B. subtilis* ATCC 23856, *B. subtilis* ATCC 23857, *B. subtilis* ATCC 23858, *B. subtilis* ATCC 23859 & *B. subtilis* natto - and three strains of *B. licheniformis* - *B. licheniformis* NCIMB 1525, *B. licheniformis* NCIMB 6816 & *B. licheniformis* 9945a will be investigated for the production of γ-PGA.

To obtain γ-PGA with different yields and properties, two different media will be used for production. Previous research demonstrates that media composition can influence the molecular weight (Shih and Van, 2001). Our endeavour was to investigate whether properties of γ-PGA such as molecular weight, crystallinity, form of peptide and yield were dependent on media composition or on bacteria producing it or on both these factors.

In addition, this work will evaluate the protective effect of γ-PGA on the viability of probiotic bacteria under conditions where unprotected cells struggle to survive. This work will be novel since γ-PGA has not been used for maintaining viability of probiotic bacteria. An introduction to probiotics is given in **Section 2**.

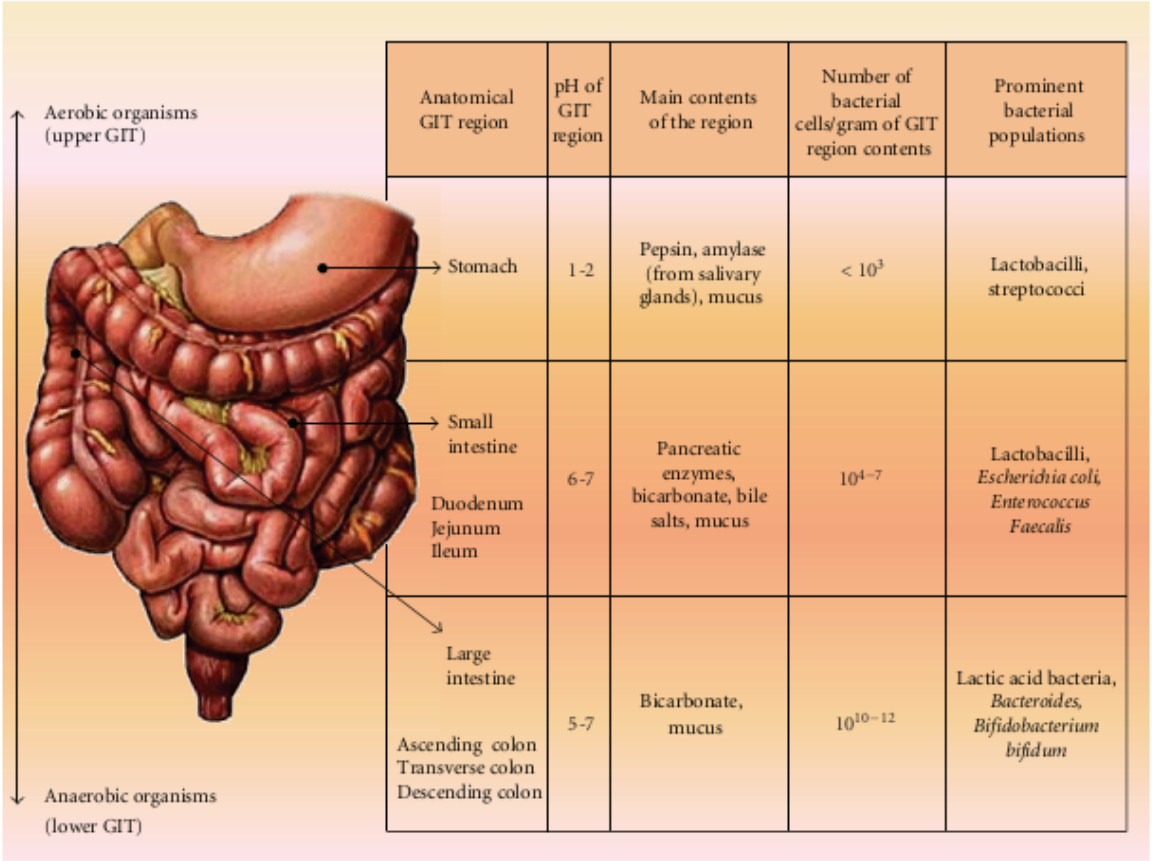
2. INTRODUCTION – PROBIOTICS

This section will describe the history and significance of probiotics. Various health benefits of probiotics will be explained and the challenges of producing a viable probiotic product will be highlighted. Some of the current solutions to these challenges will be discussed and the need to come up with a better and unique method to overcome these challenges will be illustrated.

2.1 INTRODUCTION

Promoting the use of food as medicine has existed since the time of Hippocrates, albeit without scientific backing. However, over the years, a strong relationship between diet and health has been well established (Figueroa-González *et al.*, 2011). A lot of scientific studies have highlighted the importance of functional foods for treatment or prevention of certain disorders (Allaker and Douglas, 2009; Anukam *et al.*, 2008; Figueroa-González *et al.*, 2011; Hlivak *et al.*, 2005; de LeBlanc *et al.*, 2005; Perdigón *et al.*, 1991). Although there is no precise definition for functional food, one can define it as any fresh or processed food that has an ability to promote health or prevent a disease beyond the basic function of providing nutrients. Within functional foods, probiotic food has gathered a lot of interest. Probiotics can be defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Vasiljevic and Shah, 2008; Reid *et al.*, 2003).

In total, the human gut microbiota contains about 10^{13} to 10^{14} bacterial cells (Cani and Delzenne, 2009). In fact, the number of bacterial cells in our body exceeds the number of human cells by a factor of 10 (Turnbaugh *et al.*, 2007). These bacteria are acquired rapidly after birth (Vinderola *et al.*, 2011). Different number and type of bacteria reside in different parts of the gastrointestinal tract (**Fig 2.1**).



Anatomical GIT region	pH of GIT region	Main contents of the region	Number of bacterial cells/gram of GIT region contents	Prominent bacterial populations
Stomach	1-2	Pepsin, amylase (from salivary glands), mucus	$< 10^3$	Lactobacilli, streptococci
Small intestine Duodenum Jejunum Ileum	6-7	Pancreatic enzymes, bicarbonate, bile salts, mucus	10^{4-7}	Lactobacilli, *Escherichia coli*, *Enterococcus Faecalis*
Large intestine Ascending colon Transverse colon Descending colon	5-7	Bicarbonate, mucus	10^{10-12}	Lactic acid bacteria, *Bacteroides*, *Bifidobacterium bifidum*

Fig 2.1: GIT characteristics and localization of various bacterial populations in the gut (Prakash *et al.*, 2011).

The stomach is highly acidic (pH 1-2) due to the presence of hydrochloric acid and houses less than 10^3 cells/gm of bacteria. The prominent bacterial population in this region comprises of *Lactobacilli* and *Streptococci* (Prakash *et al.*, 2011). The small intestine (duodenum, jejunum and ileum) has a higher pH (6-7). *Lactobacilli*, *E. coli* and *Enterococcus* reside in this region, with populations ranging from 10^4 to 10^7 cells/gm. The large intestine, comprising of the ascending, transverse and descending colon is the richest in terms of bacterial cells, with cell numbers ranging from 10^{10} to 10^{12} cells/gm. Bacteria living in the large intestine include lactic acid bacteria, *Bacteroides* and *Bifidobacterium* species. The bacteria in the body are present throughout our lives and undergo change in their composition

and number depending on various intrinsic (age, stress etc) and extrinsic (diet, medical treatment) factors. Of the different bacteria that colonize the gastrointestinal tract, *Bifidobacteria* and *Lactobacilli* are the most beneficial. Not surprisingly, these are chosen most often for use as probiotics, because they promote healthy effects in their natural niche. These bacteria have also been shown to decrease the occurrence of certain disorders (see **Section 2.4**), which further suggests that targeted increase in their numbers could help ameliorate disorders, thus promoting overall well-being (Prakash *et al.*, 2011).

The cost of healthcare is increasing. The elderly expect an improved quality of life. In addition, healthy living and nutrition is being given a lot of importance. There is a marked concern over side effects of some of the available medicines for age-related disorders. Due to this, naturally occurring health treatments are being heavily promoted. The western world especially looks at food today for promoting a state of well-being, improving health and reducing the risk of diseases (Granato *et al.*, 2010). Probiotics are not only used to achieve overall well-being, but also for prevention of initiation, promotion and development of non-transmissable chronic diseases (Granato *et al.*, 2010).

Probiotics can be administered in a number of forms. They can either be in the form of capsules, health food supplements or administered in food products. Dairy foods such as milk, yoghurt and yoghurt drinks are common vehicles for delivery of probiotic bacteria (Burgain *et al.*, 2011; Forssten *et al.*, 2011; Vinderola *et al.*, 2011).

2.2 SIGNIFICANCE OF FUNCTIONAL FOODS AND PROBIOTICS

Functional foods, in particular probiotics, have garnered a lot of interest, especially in developed countries and hence, their market is increasing rapidly. In 2003, the world market

for functional foods was estimated to be US$33 billion (Menrad, 2003). The European market alone was estimated to be more than US$2 billion (Menrad, 2003). According to a market survey, the average North-American consumer spent approximately US$90 per year on functional foods and beverages in 2007 (Euromonitor, 2009). Development and production of functional foods is one of the fastest growing industries and according to a market research performed by Just-Food, sales were expected to exceed $167 billion US by 2010 (Just-Food, 2004).

The global market for probiotic ingredients, supplements and foods was worth US$14.9 billion in 2007 and was US$16 billion in 2008. By 2013, it is estimated that probiotic sales would reach US$ 19.6 billion. Interestingly, *Lactobacillus* accounted for the largest share in sales of probiotic bacteria, representing 61.9% of total sales in 2007 (Granato *et al.*, 2010). In Europe, the probiotic food and beverage market is expected to rise from its 2006 position of US$ 61.7 million to US$ 163.4 million by 2013 (Granato *et al.*, 2010).

2.3 HISTORY OF PROBIOTIC DEVELOPMENT

Although scientists like Escherich (1885), Moro (1900) and Tissier (1908) initially recognized the importance of studying bacteria appearing in the faeces and the intestinal tract of humans and the difference between breast fed and bottle fed infants with respect to their intestinal bacterial population, it was Metchnikoff who first observed the benefits of microorganisms on human beings (Vasiljevic and Shah, 2008). He observed that Bulgarian peasants had an exceptionally long life span (87 years). He soon attributed this to the consumption of large amounts of fermented milk in their diet. He then proposed the auto-intoxication theory (Metchnikoff, 2004) which states that pathogens in the body continuously produce toxins, thereby weakening the immune system and our body's resistance. According

to Metchnikoff, this could be prevented by the consumption of sour milk and lactic acid producing bacteria. He studied this further and proposed that *Lactobacillus bulgaricus* can launch into the intestinal tract and prevent putrefactive effects by reducing the number of putrefactive bacteria in the intestine. However, this was later refuted by Herter and Kendall (Herter and Kendall, 1908).

Even though Metchnikoff's theory was challenged, other scientists had already shown interest in investigating health benefits conferred by other microorganisms. Probiotics became more promising owing to growing research on microorganisms with respect to their health benefits on humans. They managed to attract commercial attention in products fermented by *L. acidophilus* (Burke, 1938). In Japan in 1930, Minoru Shirota succeeded in isolating and culturing a *Lactobacillus* strain capable of surviving the passage through the gastrointestinal tract. The culture identified as *Lactobacillus casei* strain Shirota was successfully used for the production of the fermented dairy product called "Yakult", which initiated the foundation of the company with the same name in 1935. In the late 1930s to late 1950s, research on probiotics starting fading away, possibly due to the difficulties faced by the world during those times, such as depression and war. However, there was rejuvenated interest in the late 1950s and early 1960s, which led to the introduction of the probiotic concept (Vasiljevic and Shah, 2008). By 2004, there were more than 70 probiotic-containing products in the market worldwide.

For simplicity, probiotic can be thought of as an antonym to antibiotic. Vergin (1954) used the term probiotic in reference to a substance that could be used to restore the microbial imbalance in the body that was caused after an antibiotic treatment (Vergin, 1954). This is thought to be the first reference to probiotics as they are defined nowadays. Following this

many researchers defined probiotic in their own way (Fujii, 1973; Kollath, 1953; Lilly and Stillwell, 1965; Parker, 1974). In 2002, the WHO/FAO defined probiotics as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Vasiljevic and Shah, 2008; Reid *et al.*, 2003). A more recent definition of probiotics is provided by Fric (2007), who states that "probiotics are non-pathogenic micro-organisms, mostly of human origin, which confer a health benefit on the host and enable to prevent or improve some diseases when administered in adequate amounts" (Fric, 2007).

There are a wide variety of microorganisms with potential probiotic properties. However, the bacteria that stand out for their role as probiotic microorganisms conferring a health benefit on the host are *Lactic Acid Bacteria* (LAB) and *Bifidobacteria*. LAB are Gram positive, acid tolerant, strictly fermentative bacteria that produce lactic acid as their main product (Stiles and Holzapfel, 1997). The advantage of using lactic acid bacteria is that they can convert sugars into other carbohydrates and lactic acid. This provides the characteristic sour taste of fermented dairy foods such as yogurt but also the low pH makes sure that undesirable microorganisms fail to grow and hence this could be instrumental in preventing gastrointestinal infections. *Bifidobacteria* are Gram-positive, anaerobic, rod shaped bacteria. They have been shown to play important roles in human health by increased humoral immunity and maintaining balance of intestinal microflora (De Simone *et al.*, 1992; Benno and Mitsuoka, 1992). The population of *Bifidobacteria* in the human gut decreases with age and is affected by other external factors (Bornside, 1978). Therefore, it is important to replenish the population of these bacteria using probiotics. Both LAB and *Bifidobacteria* have been incorporated in various dairy and other food products, being commercially available in milk, sour milk, fruit juices, ice cream, single shots and oat-based products.

2.4 HEALTH BENEFITS FROM PROBIOTICS

Over the years, extensive research has been done to determine the efficacy of probiotic foods in controlling and alleviating disorders and diseases. Tests have been performed using animals as well as human volunteers. Overall, it has been seen (**Fig 2.2**) that probiotics can exert their beneficial effects by 5 different mechanisms (Prakash *et al.*, 2011; Rolfe, 2000).

1) Production of pathogen inhibitory substances: Probiotic bacteria can produce substances (organic acids, hydrogen peroxide or bacteriocins) that can inhibit the growth/metabolism of pathogenic bacteria and can also reduce toxin production.

2) Blocking of adhesion sites: Some probiotic bacteria can adhere to epithelial cells, where they can competitively inhibit bacteria from attaching to the intestinal epithelial surfaces.

3) Nutrient competition: Probiotics may consume nutrients that would otherwise be utilized by other bacteria, thus challenging their survival.

4) Degradation of toxins and toxin receptors: Probiotics can also degrade the toxin receptors for bacteria, such as *C. difficile*, on the intestinal mucosa, rendering them ineffective.

5) Modulation of immune responses: Stimulation of specific and non-specific immunity by probiotic bacteria is another mechanism by which protection against intestinal disorders could be sought.

The numerous health benefits of probiotics will be discussed in the following sections.

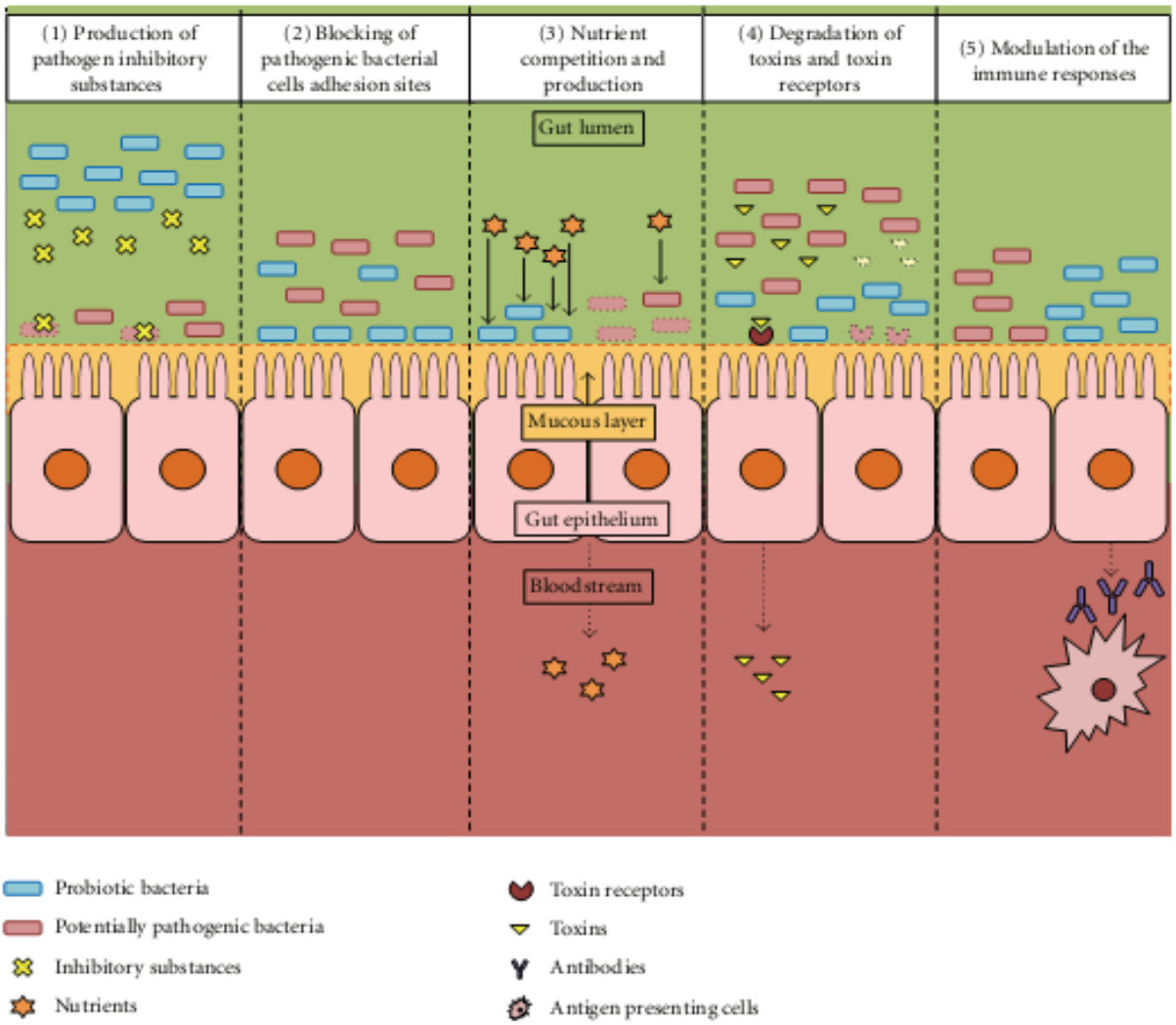


Fig 2.2: The five mechanisms by which probiotic bacteria can positively influence human health (Prakash *et al.*, 2011).

2.4.1 Effect against gastrointestinal disorders

Researchers have demonstrated the role of probiotics in alleviating several gastrointestinal disorders and promoting healthy digestive functions (Garrait *et al.*, 2009; Hammerman and Kaplan, 2006; Kligler *et al.*, 2007). This is achieved by competitively inhibiting and excluding pathogens (Figueroa-González *et al.*, 2011). For instance, probiotics have been used for eradication of primary and recurrent infection by *Clostridium difficile* (Pochapin, 2000). In this study, the bacteria that proved to be useful for control and treatment of *C. difficile* infection were *Lactobacillus rhamnosus* GG and *Saccharomyces boulardi*.

The use of probiotics to cure Ulcerative Colitis (UC) has also been investigated (Cui *et al.*, 2004). Capsules of *Bifidobacteria* were administered to patients suffering from UC after treatment with a UC standard therapy. Only 20% of the patients that were given the probiotic suffered a disease relapse in comparison with 93.3% patients in the control group.

In another study, the effect of probiotics on the eradication of *Helicobacter pylori* (HP) was studied (Park *et al.*, 2007). HP infections are common among individuals, especially in tropical countries. Usually, these infections are treated using a triple antibiotic therapy. In this study, 352 HP infected individuals were divided randomly into two groups. One group received treatment containing triple-plus probiotics (a mixture of *B. subtilis* and *Streptococcus faecium*) and the control received triple-only treatment. The patients were evaluated for four weeks. The triple-plus-probiotics group showed a higher eradication rate than the triple-only group (83.5% as opposed to 73.3%). The triple-only group showed more overall side effects and diarrhoea, demonstrating that probiotic bacteria can help with the treatment of HP infections and alleviate side effects of the triple antibiotic therapy.

In an interesting study, twenty four Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) patients with clinical signs of moderate diarrhoea, CD4 counts over 200 and not receiving any antiretrovirals or dietary supplements were administered with conventional yogurt (fermented with *L. delbruekii* var bulgaricus and *Streptococcus thermophiles*) supplemented with *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 (Anukam *et al.*, 2008). 12 patients were given supplemented product and the other 12 were given unsupplemented yogurt (control group). Diarrhoea, flatulence and nausea were cured in 12/12 probiotic treated subjects within two days compared to 2/12 receiving unsupplemented yogurt for 15 days. It was shown the probiotic

yogurt can be used to improve the quality of life of women in Nigeria with HIV/AIDS (Anukam *et al.*, 2008).

In another study, administration of *L. rhamnosus* strains shortened the duration of rotaviral diarrhoea in children (Szymanski *et al.*, 2006). Intervention shortened the time needed for intravenous rehydration. A similar study was conducted a year later which showed that rotavirus was challenged by probiotic *L. rhamnosus* GG (Pant *et al.*, 2007). When it was used in combination with immunoglobulins, the diarrhoea outcomes were shown to reduce significantly. The histopathological changes (swollen villus tips and vacuolization) were prevented and the virus load on the intestines was also reduced. Due to the use of probiotics, the treatment procedure was seen to be quicker and more cost efficient. Also, probiotics do not require special storage conditions like vaccines and hence, it has the potential to complement the rehydration therapy that is being currently used in areas where storage and transport of vaccines is challenging (Pant *et al.*, 2007).

Diarrhoea (developed after administration of antibiotics) in two oncology patients was treated with the help of probiotics and this may therefore offer a way to bring about resolution in antibiotic-associated chronic diarrhoea (Benchimol and Mack, 2004). Another study (Delia *et al.*, 2007) also suggested that probiotic lactic acid-producing bacteria are an easy, safe, and feasible approach to protect cancer patients against the risk of radiation-induced diarrhoea.

2.4.2 Effect against infections

Probiotics can also be used to suppress, prevent or treat infections by modulating the immune system. For instance, oral administration of *Bifidobacterium breve* was seen to provide protection against rotavirus or influenza virus by activating the humoral immune system

(Kaur *et al.*, 2002). The bacteria were seen to augment anti-rotavirus IgA production or anti-influenza virus, IgG production, thus preventing the respective infections. When infants were fed with these bacteria, a significant reduction in the frequency of rotavirus shedding in stool samples was observed. Enhancing the immune function by administering *L. coryniformis* CECT5711 and *L. gasseri* CECT5714 to improve defence against gastrointestinal aggressions and infections has also been demonstrated in 30 healthy children in the age group of 3-12 (Lara-Villoslada *et al.*, 2007).

Researchers have also shown (Falagas *et al.*, 2006a) the effectiveness of probiotics for prevention of urinary tract infections (UTI). UTIs occur majorly because of the lack of presence of healthy *Lactobacilli* in the vagina which are normally present in healthy pre-menopausal women. Hence, there is a possibility of curing UTIs by using probiotics to replenish the absent *Lactobacilli*. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 seemed to be the most effective among the studied bacteria for the prevention of UTIs. The same group of researchers have also suggested that probiotics such as *L. acidophilus, L. rhamnosus* GR-1 and *L. fermentum* RC- 14 can help control vulvovaginal candidiasis (VVC) when administered orally or intravaginally (Falagas *et al.*, 2006b). VVC is a common infection in women. It is thought to occur due to lack of presence of appropriate number of *Lactobacilli* in the vagina or with presence of H_2O_2-non-producing vaginal *Lactobacilli*. It is thought to work by preventing the colonization and infection of the vagina by *Candida albicans.* Probiotics could also be used for the treatment of bacterial vaginosis (BV) (Falagas *et al.*, 2007). However a small number of clinical trials are not sufficient to support this and hence more work needs to be done to be certain of the effectiveness of probiotics on VVC and BV.

A systematic review, assessing the effectiveness of various probiotic bacteria (*Lactobacillus* strains, combinations of *Lactobacillus* and *Bifidobacterium* strains, *Bifidobacterium* strain and a non-pathogenic *Enterococcus faecalis* strain) for treatment of Respiratory Tract Infections (RTIs), has been conducted (Vouloumanou *et al.*, 2009). In 14 randomised controlled trials, it was concluded that probiotics may have a beneficial effect on the severity and duration of symptoms of RTIs but probiotic treatment does not appear to reduce the incidence of RTIs. The administration of probiotics also appeared to have a good safety profile with mild or no side effects on the individuals.

2.4.3 Effect against cancer

There is substantial experimental evidence to suggest that probiotics may be beneficial in the prevention and treatment of various types of cancer. LAB have shown antitumour activity in murine breast cancer (de LeBlanc *et al.*, 2005). A study demonstrated that 7 days of cyclical administration of milk fermented by 2 strains of *L. helveticus* diminishes tumour growth, stimulating an antitumour response. It was seen to modulate the relationship between the immune and endocrine systems, which has a crucial effect on oestrogen-dependent tumour and induced cellular apoptosis. In another study (de Moreno de LeBlanc *et al.*, 2007), treatment of colon cancer and breast cancer has been shown to benefit from the use of LAB. The mode of action of LAB was thought to be due to their suppressive action against bacteria that convert procarcinogens into carcinogens, hence reducing the amount of carcinogens in the intestine.

The mortality rate of rats suffering from 1,2-dimethylhydrazine induced colon cancer reduced when they were fed with *S. thermophilus*-fermented skim milk (Shackelford *et al.*, 1983). In another study, Geier *et al.* (2006) showed that probiotics and prebiotics have the potential to

impact significantly on the development, progression and treatment of colorectal cancer and may have a valuable role in cancer prevention (Geier *et al.*, 2006). *Bacillus polyfermenticus* SCD was seen to have strong adherent properties in the colon and also have anticarcinogenic effects *in vitro* and *in vivo* (Lee *et al.*, 2007). In 1995, a study showed *L. casei* to be effective in preventing the recurrence of superficial bladder cancer (Aso *et al.*, 1995). *B. longum* has also been shown to inhibit carcinogen induced colon cancers and precursor lesions in several studies (Singh *et al.*, 1997; Reddy and Rivenson, 1993).

2.4.4 Other health benefits

The effect of *Enterococcus faecium* M-74 enriched with selenium was investigated on the levels of total cholesterol in 43 volunteers (Hlivak *et al.*, 2005). The subjects were divided into 2 groups and the group that was administered with the bacterium for 56 weeks showed a marked reduction (by 12%) in total serum cholesterol as opposed to the placebo group, which did not show any significant change in levels of cholesterol. This proved that probiotic bacteria could be used to alleviate disorders linked to high cholesterol in the blood. The researchers believe that probiotics will surely find their place as a therapeutic alternative in human medicine.

In addition, research has shown that probiotics can be used to cure dental disorders. *L. rhamnosus* CG, *L. casei, L. reuteri* and *Bifidobacterium* DN-173010 have all demonstrated the potential to alter colonisation of cariogenic bacteria and thus prevent dental caries (Allaker and Douglas, 2009). Krasse *et al.* in 2006 showed that gingivitis can be cured with the help *L. reuteri*, where a reduction in plaque levels and gingival inflammation was seen. There appeared to be two modes of action (direct and indirect) for the alleviation of dental diseases. Microbes could directly interact with the dental plaque, where they could compete

for binding sites on host tissues and for nutrients with the microbes already present in the plaque biofilm. Bacteria like lactic acid bacteria produce a range of antimicrobial agents which include organic acids, hydrogen peroxide, low-molecular weight antimicrobial compounds, bacteriocins and adhesion inhibitors, which give them a competitive advantage. Indirectly, the probiotic bacteria can alter the innate and specific immune function which could help alleviate the dental disorder (Allaker and Douglas, 2009).

2.5 MAINTAINING PROBIOTIC VIABILITY

As previously mentioned (see **section 2.4**), there is a lot of evidence in the literature about the benefits of probiotics. However, there remains an uncertainty about the usefulness of these bacteria. Some data showing positive effects may be irreproducible and one of the reasons for this could be insufficient/inconsistent numbers of live bacteria reaching the intestine. Many different probiotics have been defined by researchers (for some examples, see **section 2.3)**. Most of the definitions used for probiotics stress the importance of "adequate" numbers of administered bacteria. The benefits of probiotic bacteria are largely dependent on their ability to survive, colonize and multiply in the host (Anal and Singh, 2007; Burgain *et al.*, 2011). If enough viable bacteria do not reach the target site, the probiotic product would not be useful. Many of the probiotic microorganisms used in food and drink products do not survive for long enough to confer a health benefit on the host. Several review articles on probiotics have mentioned studies that highlight the loss in viability of probiotic bacteria during different stages in their manufacture, storage and after ingestion (Kailasapathy and Chin, 2000; Figueroa-González *et al.*, 2011). Therefore, the major challenge for administering a useful probiotic product is to maintain viability, which is a prerequisite for achieving health benefits via microorganisms. Recently, the importance of improving probiotic viability during the

manufacturing process and storage of functional foods has been highlighted (Figueroa-González *et al.*, 2011).

It has been established by WHO/FAO that any food sold with health claims based on the addition of probiotics must contain at least 10^6 to 10^7 CFU/ml of viable probiotic bacteria (Kailasapathy and Chin, 2000; Kurmann and Rasic, 1991). There are several stages where the viability of probiotic bacteria is susceptible. Firstly, the bacteria have to survive the processing stage. Following this, if the probiotic bacteria are to be administered in food, they have to endure the storage period or shelf life of the food in which they are delivered (Forssten *et al.*, 2011). Finally, upon ingestion, they have to survive the acidic conditions of the stomach as well as the bile salts in the small intestine, before reaching the lower portions of the gastrointestinal tract where they will provide beneficial effects (Kailasapathy and Rybka, 1997). Some of the challenges encountered will be discussed in more detail in the following sections.

2.5.1 Viability during freeze drying

It is common practice to freeze dry bacteria, so that they can be stored in the form of dry powders for a longer period of time. In addition, transportation of freeze dried bacteria is easier. Freeze drying is based upon sublimation and occurs in three phases – freezing, primary drying and secondary drying (Meng *et al.*, 2008). Probiotic cultures are frequently supplied as freeze-dried or spray-dried powders. However, the procedures used to prepare freeze dried probiotic bacteria are detrimental to cell structure and viability (Saarela *et al.*, 2005). Freeze drying can cause protein denaturation and DNA damage that leads to a decrease in cell viability (Carvalho *et al.*, 2004). Also, desiccated bacteria could lose viability on rehydration (Peccia *et al.*, 2001). The cell membrane has been reported to be the major site

of damage during drying or rehydration (Santivarangkna *et al.*, 2008). The viability of lactic acid bacteria has been assessed before and after freeze drying (Jagannath *et al.*, 2010). In the study, it was observed that the viability decreased by 3 log CFU/g (from 10^{10} CFU/g to 10^{7} CFU/g) immediately after the freeze drying process.

2.5.2 Viability during storage

The viability of probiotic bacteria in food products decreases due to exposure to detrimental environmental factors such as organic acids, hydrogen ions, molecular oxygen and antibacterial components (Mortazavian *et al.*, 2007; Sultana *et al.*, 2000; Vinderola *et al.*, 2000). Many researchers have tried to enumerate bacteria (*L. acidophilus* and *Bifidobacterium*) in commercial probiotic foods, only to find that the levels are either not consistent or are very low (Huys *et al.*, 2006; Masco *et al.*, 2005; Micanel *et al.*, 1997; Tharmaraj and Shah, 2003; Vinderola *et al.*, 2000). The concentration of microorganisms in the probiotic products fail to correspond to the information on the label. Kailasapathy and Rybka (1997) observed that at the expiration date (4 weeks) of yoghurt, the contents of *L. acidophilus* and *B. bifidum* were lower than the levels on the label (10^{6} CFU/g), indicating failure to survive (Kailasapathy and Rybka, 1997). A pH value of 4.5 or lower was detrimental to cell viability of the probiotic organisms in yoghurt, which was stored at 5°C. In a study to determine viability of *B. longum* during refrigerated storage in yoghurt, it was found that bacterial viability decreased by 98% after 28 days (Akalin *et al.*, 2004). In fact, after 14 days, the viability of *B. longum* was 4.8 x 10^{5} CFU/g, which is lower than the recommended FAO/WHO value (10^{6} – 10^{7} CFU/g). In a different study (Christopher *et al.*, 2009), the viability of *B. bifidum* strains was evaluated in set and stirred yoghurts for 35 days. It was seen that the viability of all bacteria decreased (from 8.4-8.94 log CFU/g to 4.83-5.93 log CFU/g) at the end of the storage period. When *B. longum* was incorporated in orange

juice, cells showed rapid loss in viability over the juice's storage period (Ding and Shah, 2008). By the fifth week of storage, a complete loss in viability was seen.

2.5.3 Viability after ingestion

It is important that probiotic bacteria survive passage through the upper gastrointestinal tract and colonize the large intestine. This is especially crucial for probiotic bacteria used for treatment/prevention of diarrhoea (Figueroa-González *et al.*, 2011). It has been established that probiotic microorganisms are extremely susceptible to environmental conditions such as water activity, redox potential, temperature and acidity (Siuta-Cruce and Goulet, 2001). There is enough evidence in the literature stating that the viability of probiotics depends the most on the acidity levels of the delivery system (for example yogurt) and the stresses encountered in the gastrointestinal tract (Vasiljevic and Shah, 2008). Probiotics do not seem to thrive below pH 4.4, although this is dependent on the microorganism. Compared to *Bifidobacterium* spp., which is very susceptible to low pH levels, *L. acidophilus* is more resistant due to its high cytoplasmic buffering capacity (Rius *et al.*, 1994). The growth of *Bifidobacterium* is hampered significantly in highly acidic environments or in the presence of oxygen. When commercial *Bifidobacteria* cultures were exposed to simulated gastric fluid (pH 1.5) for 2 h, their viability decreased from 10^{11} CFU/g to $<10^{3}$ CFU/g (Cui *et al.*, 2000). In a recent study, a loss in viability of more than 1 x 10^{8} CFU/ml was observed for *B. longum* cells when they were exposed to simulated gastric juice for 2 h (Su *et al.*, 2011).

2.6 MAINTAINING PROBIOTIC VIABILITY - CURRENT SOLUTIONS

Scientists have endeavoured to use different techniques to resolve the loss of viability in probiotics. Cryoprotectants could be used to avoid/minimize loss in viability during freeze

drying. For instance, 'Unjpectine™ RS 150' has been successfully used to prevent loss in viability during freeze drying of probiotic bacteria (Capela *et al.,* 2006). Other cryoprotectants that have been used include bacterial cellulose, sucrose, trehalose, skimmed milk, glycerol, polyvinylpyrrolidone and DMSO (Palmfeldt *et al.*, 2003; Barbaree *et al.*, 1982; Panoff *et al.*, 2000; Jagannath *et al.*, 2010). These materials, although useful for certain applications, are expensive to obtain and maintain and are known to be impractical when equipment and refrigeration are not readily available (Krumnow *et al.*, 2009).

For protection of probiotic bacteria during storage and after ingestion, microencapsulation and enteric coating have been suggested and investigated (Özer *et al.*, 2009; Vasiljevic and Shah, 2008). Microencapsulation is a process where the cells are enclosed within an encapsulating membrane so that they can be protected from the environment. It can be defined as the "entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalization" (Poncelot, 2006). It provides an effective barrier and protects the cells from degradation by elements of the external environment and from various stresses present during production and/or storage. As such, it preserves the viability of the probiotic microorganism and improves its shelf life, even at room temperature (Del Piano *et al.*, 2006). Some of the encapsulating materials used are gelatine gum, vegetable gum and alginate. When encapsulated probiotic microorganisms were incorporated in fermented frozen dairy desserts, yoghurt or freeze dried yogurt, they showed improved viability in comparison to their non-encapsulated counterparts (Capela *et al.*, 2006; Ravula and Shah, 2000).

Although there is evidence of the positive effects of microencapsulation for probiotics, it is still far from being used routinely in the industry, because the current technologies used

present difficulties for scaling up (Mortazavian *et al.*, 2007; Burgain *et al.*, 2011). The process of encapsulation itself can have detrimental effects on the viability of probiotic bacteria and can cause cell damage. Microencapsulation by spray drying, for instance, is an effective and economical technology for protecting materials, however, it is rarely considered for cell immobilization because it requires simultaneous dehydration and thermal inactivation steps which lead to high cell mortality (Anal and Singh, 2007). Methods for production of beads, such as extrusion cannot be used for large scale production due to slow formation of beads (Burgain *et al.*, 2011). The emulsion method can be easily scaled up, but it is costlier than the extrusion method (Burgain *et al.*, 2011). Also, the presence of residual oil on the capsule surface is detrimental to the texture and the organoleptic properties of the product.

Encapsulation techniques are also limited by the size of beads. In their review, Anal and Singh (2007) point out that the large size of microbial cells or particles of freeze-dried culture limits cell loading for small capsules (Anal and Singh, 2007). Large capsules have undesirable effects on the textural and sensorial properties of food products to which they are added. However, reduction in capsule size may result in loss of protective ability of the capsule. For instance, it has been reported that very large calcium alginate beads (>1 mm) change the texture in live microbial feed supplement (Hansen *et al.*, 2002). In addition, small beads (< 100 µm) are unable to significantly protect bacteria in simulated gastric juice when compared to unprotected cells. In some studies, it is evident that although microencapsulation of *Bifidobacteria* and *Lactobacillus* spp. with calcium alginate improves survival during refrigerated storage, it does not cause a marked improvement in their survival when exposed to highly acidic environments such as simulated gastric juice (Chandramouli *et al.*, 2004; Hansen *et al.*, 2002; Anal and Singh, 2007; Mortazavian *et al.*, 2008; Krasaekoopt *et al.*, 2004). In fact, in low pH conditions, the beads are known to significantly shrink in size and

exhibit decreased mechanical strength. In a recent study, after incubation in simulated gastric juice (pH 2.0, 2h), *B. longum* encapsulated in microspheres made from 1.5% (w/v) alginate - 2% (w/v) human-like collagen showed a decrease in viability of 4.66 log CFU/ml (Su *et al.*, 2011). The total number of live bacteria after 2 h incubation in simulated gastric juice was 4.81 log CFU/ml, lower than the recommended FAO value (6-7 log CFU/ml).

Burgain *et al.* (2011) have highlighted the advantages and disadvantages of using different materials for encapsulation, such as alginate, gellan gum, κ-carrageenan, chitosan, starch, etc (Burgain *et al.*, 2011). For instance, κ-carrageenan beads keep the bacteria in a viable state but the produced gels are brittle and are not stress resistant (Chen and Chen, 2007). In addition, it needs potassium ions for structural stabilization, which is harmful for the body in high concentrations (Mortazavian *et al.*, 2007). A mixture of chitosan with alginate is a good coating agent for reducing loss in viability of probiotic bacteria. However, it was seen to have inhibitory effects on Lactic acid bacteria (Groboillot *et al.*, 1993). Starch has been shown to offer good protection to probiotic bacteria. In addition, because of its prebiotic functionality, starch can also be utilized by probiotic bacteria in the large intestine (Mortazavian *et al.*, 2008). However, starch was seen to enhance probiotic viability in the product, but not under simulated gastrointestinal conditions (Sultana *et al.*, 2000). Moreover, starch is a polysaccharide made of up of a large number of glucose units joined together. Therefore, a health-promoting probiotic product containing starch is not ideal, especially for diabetic patients. Also, substances such as alginate, κ-carrageenan, gellan-gum or xanthan cannot be used as coating materials in dairy products in certain countries (Picot and Lacroix, 2004).

Although microencapsulation has provided significant advancements in the field of probiotics, a number of issues regarding the formulation of a microencapsulated probiotic

product need to be addressed (Prakash *et al.*, 2011). It is apparent from the studies mentioned above that a convincing solution to the problem of loss in viability of probiotics is yet to be achieved.

Alternatively, the viability of probiotics in the product and subsequently in the gastrointestinal tract can be improved by addition of an appropriate prebiotic. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve health" (Gibson and Roberfroid, 1995). Prebiotics such as 'Raftilose®P95' have been successfully used to improve the viability of probiotics in yogurt (Capela *et al.*, 2006).

Shima *et al.* (2009) have come up with another way of preventing loss of viability of probiotic organisms due to the environmental stresses in the gastrointestinal tract (Shima *et al.*, 2009). They found that incorporation of *L. acidophilus* in the inner-water phase of a water/oil/water (W/O/W) emulsion significantly improved the cell viability compared to free cells, when they were incubated in a model stomach solution for 2 h.

Recently, chocolate matrices have also been used as delivery agents for probiotic bacteria (Possemiers *et al.*, 2010). Dark and milk chocolates were evaluated for their protective effect on the viability of probiotic bacteria during their passage through the stomach and small intestine. Milk chocolate offered better protection to the cells (91% and 80% survival for *L. helveticus* and *B. longum* respectively) than dark chocolate, when they were passed through simulated gastric and intestinal juices for 3.5 h, and this was thought to be due to the presence

of 5-fold lower polyphenol content in milk chocolate. However, using milk chocolate as a delivery vehicle for probiotic bacteria might not be ideal for diabetic patients.

As is evident from the literature, scientists are putting in considerable effort to come up with an optimum technology to avoid loss in viability of probiotic cultures due to different stresses, so that its usefulness in conferring health benefits to the consumer can be improved. It has been stated that the current commercial expansion in the use of probiotic bacteria in functional foods must be accompanied by an in depth understanding of the different factors inherent to technological process, the physicochemical environment of food and the gastrointestinal tract that affect viability and functionality of bacteria, along with new knowledge and exploration of new concepts to tackle these problems (Vinderola *et al.*, 2011).

2.7 NON-DAIRY PROBIOTIC PRODUCTS

According to a report, 78% of current probiotic sales in the world are delivered through yoghurt (Granato *et al.*, 2010). It is important to come up with probiotic delivery vehicles other than dairy products for those with lactose intolerance or with an allergy to milk proteins (Saarela *et al.*, 2005). Identification of non-dairy probiotic foodstuffs to administer probiotic bacteria has an additional advantage, since these products may have reduced cholesterol levels when compared to dairy products (Granato *et al.*, 2010). Recently, several such food products have been researched (Khalf *et al.*, 2010; Ding and Shah, 2008; Possemiers *et al.*, 2010). Fruit juices could be attractive delivery agents for probiotic bacteria because they inherently contain essential nutrients, they are "good looking" and have good taste (Luckow and Delahunty, 2004; Sheehan *et al.*, 2007). However, fruit juices provide a less suitable environment for survival of probiotic bacteria. Viability of *Bifidobacteria* has been shown to be poorer in fruit juice than in milk (Saarela *et al.*, 2006). One of the aims of this study was to

explore the use of γ-PGA as a delivery tool for probiotics in an attempt to improve survival of bacteria in orange and pomegranate juice during storage.

2.8 USE OF γ-PGA WITH PROBIOTICS

To our knowledge, γ-PGA has not been used for maintaining viability of probiotic bacteria during different stages of production, storage and ingestion. This work will utilize γ-PGA for a novel probiotic application where the effect of γ-PGA will be tested on the viability of probiotic bacteria during freeze drying, during storage in fruit juice and in simulated gastric juice.

As previously discussed (see **sections 1.1 & 1.5.1**), γ-PGA has unique properties. It is edible, non-toxic and biodegradable. It has been shown to be stable at low pH and to dissolve readily at higher pH (Agresti *et al.*, 2008). Since some of the major challenges for probiotic products is the survival of beneficial microorganisms in the low pH environments during extended cold storage and in the stomach, there is enough evidence to hypothesize that if probiotics are administered with γ-PGA, they will be protected from the deleterious effects of both of these. In the stomach, which is an environment of high acidity, γ-PGA would remain intact, thus masking the organisms from the surroundings. In the intestine, which is an environment of higher pH levels, γ-PGA would dissolve, thus delivering the organisms to the target site intact.

Probiotic products, especially yoghurt, undergo freeze drying to lengthen preservation. However, freeze drying has been known to decrease viability and this has been successfully prevented with the use of cryoprotectants. It is a well-known fact that γ-PGA can be used as a

cryoprotectant (Mitsuiki *et al.*, 1998; Mizuno *et al.*, 1997; Shih *et al.*, 2003). This application of γ-PGA could also be exploited with probiotics.

Dairy products that deliver probiotics are also a rich source of calcium. People who are lactose intolerant also show an inadequate ingestion of calcium and other milk provided nutrients, since they do not consume appropriate amounts of dairy products. This increases the probability of developing bone disorders such as osteoporosis. Hence, it would be ideal if the delivery agent in a non-dairy probiotic product could improve calcium absorption in the intestine. Tanimoto and co-workers (2001) have demonstrated that γ-PGA accelerates the absorption of minerals in the rat small intestine (Tanimoto *et al.*, 2001). Later, they also used human volunteers to prove that calcium absorption is better when it is administered with γ-PGA (Tanimoto *et al.*, 2007). Therefore, if γ-PGA is used in conjunction with calcium (or other minerals) and probiotics, it can possibly confer better health benefits on the consumer (for example, prevention or treatment of some calcium deficiency disorders).

There is also concern over change in palatability of fruit juices when probiotic bacteria are introduced in them (Granato *et al.*, 2010). γ-PGA has been used as a bitterness relieving agent (Sonoda *et al.*, 2000) and it could help mask the change in taste of fruit juices with probiotic bacteria, thus improving drinkability.

Another reason for the loss in viability of probiotics could be the induction of the hosts immune system upon ingestion of probiotic bacteria, which could potentially attack the delivered cells (Prakash *et al.*, 2011). Protection of cells with γ-PGA could help to shield the cells from the body's immune system, since it is known that bacteria such as *B. anthracis* and *S. epidermidis* use γ-PGA in the capsule to escape the immune response of the host (Candela

and Fouet, 2006; Candela *et al.*, 2005; Kocianova *et al.*, 2005; Makino *et al.*, 1989; Mesnage, 1998; Zwartouw and Smith, 1956).

While choosing a substance to protect probiotic bacteria, it is important to ensure that it helps maintain viability during all the stages where viability could be affected – freeze drying, during storage and after ingestion. The effect of γ-PGA on the viability of probiotic bacteria during these three steps will be evaluated.

2.9 AIMS OF THIS STUDY

The aims of this study are to:

1. Investigate production of γ-PGA in shake flasks using two media. Eight bacteria will be used, six of which have not been investigated previously.
2. Characterize the γ-PGA produced by each organism with respect to molecular weight, yield, form (salt or acid) and crystallinity using different analytical techniques.
3. Recognize factors affecting properties of γ-PGA.
4. Identify and choose the bacterium and medium that would be appropriate to produce γ-PGA for probiotic tests.
5. Use the chosen bacterium and medium to produce γ-PGA using 5 l fermentations.
6. Test the effect of γ-PGA on the viability of probiotic bacteria during freeze drying.
7. Test the effect of γ-PGA on the viability of probiotic bacteria during incubation in orange and pomegranate juice.

8. Identify changes, if any, in the organic acid content and pH of fruit juices on introduction of probiotics to ensure maintenance of organoleptic and nutritional properties of the juices.
9. Test the effect of γ-PGA on the viability of probiotic bacteria during storage in simulated gastric juice.

3. MATERIALS AND METHODS

3.1 γ-PGA PRODUCTION

3.1.1 Bacterial Strains

All bacteria (*B. subtilis* natto ATCC 15245, *B. subtilis* ATCC 23856, *B. subtilis* ATCC 23857, B. *subtilis* ATCC 23858, *B. subtilis* ATCC 23859, *B. licheniformis* 9945a, *B. licheniformis* NCIMB 1525 and *B. licheniformis* NCIMB 6816) were obtained from National Collection of Industrial and Marine Bacteria (NCIMB). The stock cultures were freeze-dried and stored at -20°C. Before use, cultures were revived aseptically and grown aerobically on general purpose microbiology media overnight at 37°C.

3.1.2 Growth Media

Tryptone soya broth (TSB), tryptone soya agar (TSA) and one-quarter strength Ringer solution were purchased and prepared according to the manufacturer's protocol (Lab M, UK). The compositions are shown in **Table 3.1 & 3.2**.

Table 3.1: Composition of TSB

Chemical	Amount
Tryptone (casein digest)	17 g/l
Soy Peptone	3 g/l
NaCl	5 g/l
K_2HPO_4	2.5 g/l
D-glucose	2.5 g/l

Table 3.2: Composition of TSA

Chemical	Amount
Tryptone (casein digest)	15 g/l
Soy Peptone	5 g/l
Agar No.2	12 g/l
K_2HPO_4	2.5 g/l
D-glucose	2.5 g/l

To determine whether the bacteria under study could produce γ-PGA in the absence of precursors, GS medium was used. Medium E contains citric acid, which is known to be the best precursor for γ-PGA production (Du *et al.*, 2005; Xu *et al.*, 2005a). Hence, this medium

was used to assess whether the bacteria could produce γ-PGA in the presence of the precursor. The composition of GS and E media has been given in **Tables 3.3** & **3.4**. The pH of GS and E media was adjusted to 7.2 using 3 M NaOH and 1 M HCl.

Table 3.3: Composition of GS medium

Chemical	Amount (g/l)	Source
L-glutamic acid	20	Sigma-Aldrich
Sucrose	50	Acros Organics
KH_2PO_4	2.7	Fisher Scientific
Na_2HPO_4	4.2	AnalaR
NaCl	50	Aldrich Chemical Co. Ltd
$MgSO_4 \cdot 7H_20$	5	AnalaR

1 ml/l of filter sterilized Murashige-Skoog vitamin solution (2.0 mg/ml glycine, 100 mg/ml myo-inositol, 0.50 mg/ml nicotinic acid, 0.50 mg/ml pyridoxine hydrochloride, 0.10 mg/ml thiamine hydrochloride) from Sigma-Aldrich was added separately to GS medium.

Table 3.4: Composition of Medium E

Chemical	Amount (g/l)	Source
L-glutamic acid	20	Sigma-Aldrich
Citric acid	12	Acros Organics
Glycerol	80	Avocado
NH_4Cl	7	Riedel-de Haën
$MgSO_4 \cdot 7H_20$	0.5	AnalaR
$FeCl_3 \cdot 6H_20$	0.2	Sigma-Aldrich
K_2HPO_4	0.5	AnalaR (BDH)
$CaCl_2 \cdot 2H_20$	0.15	Fisher Chemicals
$MnSO_4 \cdot H_20$	0.2	Sigma

All media were prepared using de-ionised water and sterilized by autoclaving at 121ºC at 1.035 BAR for 20 min. Sucrose solution was sterilised separately by autoclaving at 110ºC at

0.35 BAR for 30 min to avoid caramelization and vitamin solution was filter sterilised (0.2 µm Ministart) and added separately to GS medium.

3.1.3 Production of γ-PGA in shake flask cultures

Production of γ-PGA with each bacterial strain in shake flasks was done independently in triplicate. Bacteria producing γ-PGA usually appear to be mucoid when grown on agar plates. Highly mucoid colonies of the appropriate strain were selected and inoculated in flasks containing 250 ml TSB and incubated at 37°C for 24h. At the end of 24 h, the seed culture reached a population of ~ 7 log CFU/ml. 5% of this culture was inoculated into 250 ml of γ-PGA production medium. All flasks were incubated at 37ºC on a rotary shaker (Innova 43) at 150 rpm. γ-PGA recovery time depends on the bacteria and medium used for production. Previous research has demonstrated that γ-PGA production in *Bacillus* usually takes place in the late logarithmic and stationary phase of the culture (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007; Shi *et al.*, 2007). Hence, in this study, γ-PGA was recovered after 96 h, which is seen to be the ideal time to recover γ-PGA from the culture. 5 ml samples were taken aseptically at 0, 24, 48, 72 and 96 h for analysis of cell growth and nutrient consumption.

3.1.4 Scale-up of γ-PGA production in fermentation culture

To produce γ-PGA for probiotic tests, 4 l batch fermentations of *B. subtilis* natto were performed in a 5 l Electrolab fermenter. Highly mucoid colonies were first selected and inoculated in flasks containing 250 ml of TSB at 37°C for 24 h. The fermenter was washed thoroughly and dried. The pH probe of the fermenter was calibrated according to manufacturer's instructions. GS medium (without sucrose) was added to the fermenter, after

which the fermenter was sealed appropriately and sterilized by autoclaving at 121˚C at 1.035 BAR for 20 mins. Sucrose solution was sterilized separately by autoclaving at 110ºC at 0.35 BAR for 30 min and added to the fermenter aseptically. Acid, base and antifoam bottles were connected to the fermenter while being careful to not introduce any contamination. pH of the medium was adjusted to 6.8 with 3 M NaOH and 3 M HCl using the Electrolab automatic pH control system. The fermentation temperature was maintained at 37°C using a heating jacket. The dissolved oxygen (dO_2) probe was then calibrated according to manufacturer's protocol. Before adding the inoculum, Electrolab software was set up to monitor temperature, pH, dO_2 and agitation parameters. 5% of the inoculum was then added to the sterilized fermenter with GS medium. The stirring speed and airflow rate were set at 250 rpm and 2.0 l/min respectively at the start of fermentation. Since γ-PGA is an extracellular polymer, the culture medium becomes highly viscous with increasing polymer production. The increased viscosity decreases the volumetric oxygen mass transfer, leading to oxygen limitation. The supply of oxygen was maintained above 40% saturation by controlling the agitation speed and air flow rate.

3.1.5 Isolation of γ-PGA

After 96 h, the cell culture broth from the shake flasks or the fermenter was transferred equally (± 0.1 g) to 6 centrifuge tubes and centrifuged at 17000g for 30 minutes (Hermele 2 300 K) to separate cells from the supernatant. The cell pellets were inactivated using trigene (Medichem International, UK) before being discarded appropriately. Four volumes of cold 90% (v/v) ethanol was added to the cell free supernatant and incubated at 4°C for 72 h. Wet γ-PGA powder was obtained as sediment. The majority of the supernatant was removed using aspiration, while making sure that the sediment was not disturbed. The sediment was separated from the remaining supernatant by centrifugation at 17000g for 30 mins. The wet

crude polymer was then dissolved in water. This solution was introduced in Spectra/Por® Dialysis Membrane (MWCO: 10,000) tubes supplied by Spectrum Labs, UK. The tubes were sealed and placed in a beaker filled with deionised water at room temperature for 24 h. The water was changed at 2, 4 and 16 h to ensure a concentration gradient was maintained. This procedure eliminates impurities lower than 10,000 Da. The obtained pure polymer solution was prepared for lyophilisation in round bottom flasks. The flasks were rotated gently on a mixture of 90% (v/v) ethanol at -20ºC and dry ice to freeze the biopolymer in the form of a thin film. The frozen biopolymer was then lyophilized in an Edwards Freeze Dryer (UK), connected to an Edwards vacuum pump at -40 ºC at 5 MBAR pressure for 48-72 h to obtain dry γ-PGA powder. The pure dried powder was weighed to calculate yield in g/l and stored in a desiccator for further analysis and tests with probiotics.

3.1.6 Determination of growth

It was important to assess the growth of bacteria during the culture so that it could be compared with nutrient consumption and yield of γ-PGA obtained in different media. It would also help to ensure that the culture has not been contaminated with foreign microorganisms. Samples were taken aseptically at 0, 24, 48, 72 and 96 h to monitor cell growth. The Miles and Misra technique was used to calculate Colony Forming Units/ml (CFU/ml) in triplicate. Serial dilutions from 10^{-1} to 10^{-10} were performed by dispensing 0.5 ml of sample in 4.5 ml of sterile 1/4th strength Ringer solution. 20 µl of each dilution was dispensed onto TSA plates under aseptic conditions. The plates were then incubated aerobically at 37°C for 24 h, after which plates with less than 20 colonies were selected and used for viable count determination. Viable cell count in CFU/ml was calculated using the formula:

$$CFU/ml = n \times \frac{1}{(Sample\ volume)} \times \frac{1}{D.F.}$$

Where:
n is the number of colonies
D.F. is the dilution factor

3.1.7 Chemical analysis

3.1.7.1 Nutrient consumption analysis

It was essential to identify how the carbon and nitrogen sources were consumed by the bacteria in GS and E media. Comparing the consumption of nutrients to the growth of bacteria and yield of γ-PGA produced may provide important information of nutrient preference of bacteria. Nutrient consumption analysis was performed using High Performance Liquid Chromatography (HPLC). HPLC is a chromatographic analytical technique used for separation, identification and quantification of analytes based on selective partitioning of the molecules of interest between two different phases. The sample is introduced into the mobile phase and is passed through a column (stationary phase) using pressure. Depending on the size and interactions of the analytes present in the sample with the column, the analytes are eluted at different times. The characteristic retention times of the analytes and the chromatogram obtained are used for identification and quantification.

An HP series 1100 HPLC machine at University of Reading (Department of Food and Nutritional Sciences), U.K. was used for analysis. A Prevail Organic Acid 5µm column with a UV detector was used for analyzing L-glutamic acid whereas a Phenomenex carbohydrate column (Rezex RCM - monosaccharide Ca^{2+} - 8%) was used for sucrose and glycerol determination. Filtered deionised water was used as the eluent for sucrose/glycerol analysis. For organic acid analysis, 25 mM KH_2PO_4 (pH 2.5 adjusted with phosphoric acid) was used

as the eluent. Before loading, the samples were filtered using 0.45 µm filters and were diluted 10 fold with filtered deionised water.

To identify and quantify L-glutamic acid, sucrose and glycerol in the samples, known concentrations of these analytes were first used to prepare standard curves (**Fig 3.1**).

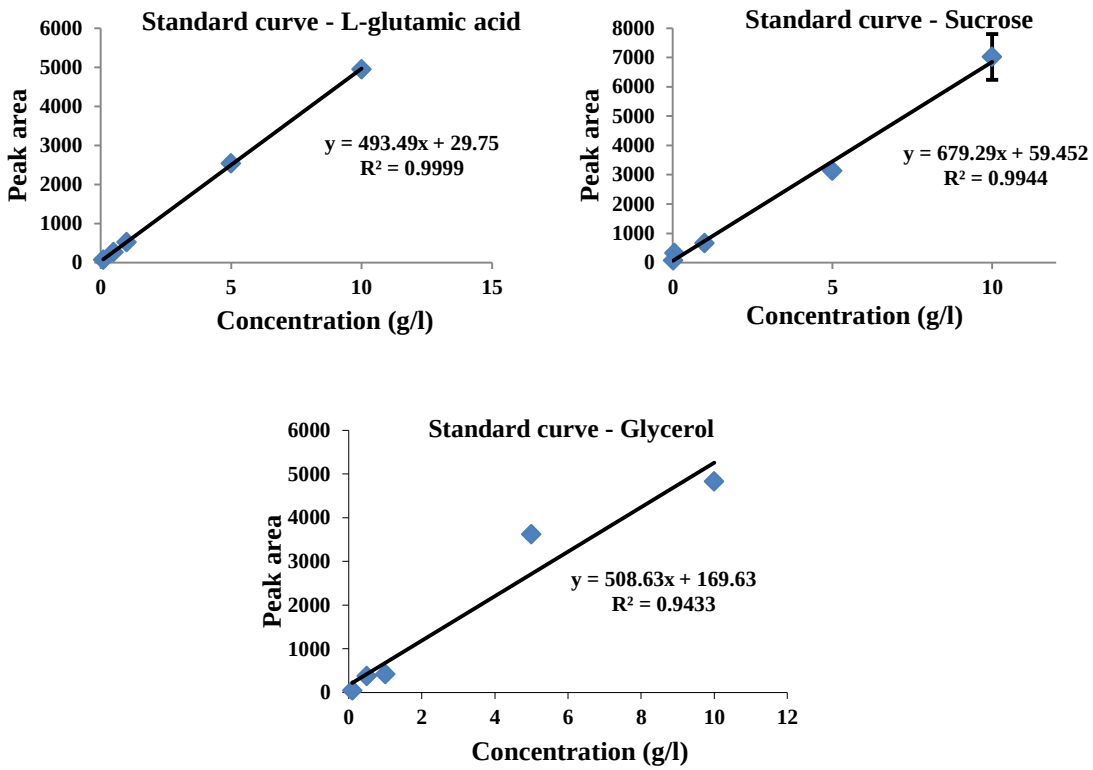


Fig 3.1: L-glutamic acid, sucrose and glycerol standard curves for nutrient consumption analysis using HPLC. Some standard error bars are too small to be seen.

3.1.7.2 Identification of γ-PGA

It was important to identify the recovered polymer as γ-PGA. Isolated biopolymer was analysed using Fourier Transform InfraRed spectroscopy (FT-IR). FT-IR is a quick, non-destructive technique and provides precise measurement which requires no external calibration. This technique involves infrared radiation being passed through the sample. The radiation is absorbed or transmitted by the sample. The resulting spectrum represents the

molecular absorption and transmission and can be considered to be a molecular fingerprint of the sample. Every molecular structure yields its unique spectrum and 2 distinct molecular structures will never produce the same infrared spectrum. Each absorption peak on the FT-IR spectrum represents the frequencies of vibrations between the bonds of the atoms making up the material. The FT-IR spectra can be used as a qualitative analysis tool to identify unknown materials and determine the quality or consistency of the sample.

An Impact 404 Nicolet spectrometer (UK) with KBr pellet in conjunction with OMNIC software was used for γ-PGA produced by bacteria in GS medium. A background scan was first performed with no sample. Preparation of sample for FT-IR analysis was performed by mixing 0.5 g of γ-PGA thoroughly with 0.5 g KBr using a mortar and pestle. A hydraulic press (Graseby Specac) was used to produce a sample disc, which was then placed in the sample holder and scanned. The FT-IR spectra of produced γ-PGA were compared with the spectra of a commercially available γ-PGA sample (Crescent Innovations Inc.).

For γ-PGA produced by bacteria in medium E, a Genesis II FTIR™ (UK) was used. This instrument does not need any sample preparation. 0.5 g of finely ground γ-PGA was placed onto the diamond attenuated total reflector. A background scan was performed after which, the sample was scanned to produce spectra.

3.1.7.3 Elemental analysis of γ-PGA

γ-PGA produced by bacteria in different media could be either in the form of a salt or free acid or a mixture of the two. To assess the percentages of different salt forms and free acid form of γ-PGA, elemental analysis was performed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). ICP-AES is an analytical technique used for the detection

of elements in a compound. It involves the use of inductively coupled plasma that produces excited atoms and ions which emit electromagnetic radiation at wavelengths characteristic of a particular element. The intensity of this emission is representative of the concentration of the element within the sample. SPECTRO CIROS^{CCD} was used for ICP-AES analysis.

Sample preparation is crucial for ICP-AES. First, the γ-PGA sample needs to be dissociated into its component elements. Therefore, prior to the analysis, 6 ml HNO_3 (70%) and 1 ml H_2O_2 (30% w/v) was added to 0.25 g of dried γ-PGA. The volume was made up to 25 ml and this solution was digested using an ETHOS 900 Microwave Labstation (Milestone Microwave Laboratory System) using a previously fed program. For ICP-AES analysis, the sample was aspirated using a sipper probe connected to a peristaltic pump. The sample was then scanned for a range of elements and the concentration of each element in the sample was represented in ppm. The concentration of the elements obtained after ICP-AES analysis represents the salt form of γ-PGA of that particular element. The concentration of salt form in percentage (from ppm) was calculated as follows:

$$\%\ \text{content of salt form of } \gamma\text{-PGA} = \frac{(M_w \text{ of } \gamma\text{-PGA salt}) \times (\text{Concentration of element in ppm})}{(M_w \text{ of element}) \times 10^6} \times \text{D.F.} \times 100$$

Where:
M_w is the molecular weight
D.F. is the dilution factor

3.1.7.4 Molecular weight of γ-PGA

Knowledge of γ-PGA molecular weight is crucial since it can directly determine the application for which γ-PGA is used. Conventional aqueous based gel permeation chromatography (GPC) was used to determine molecular weight (M_w), molecular number (M_n) and polydispersity index (Pd). Pd is the measure of distribution of molecular mass in a

given polymer sample and is calculated by the formula: $Pd = \frac{M_w}{M_n}$. Pd can have a value ≥ 1.

The closer the value of Pd to unity, the more homogenous the polymer is with respect to its molecular weight. GPC separates analytes on the basis of their size. Analytes are made to pass through porous beads packed in a column. The smaller analytes can easily enter the pores and hence spend more time in the column. This increases their retention time. In contrast, larger analytes cannot enter the pores as efficiently, and hence, their retention time is much shorter.

Molecular weight analysis was performed at Smithers Rapra in Shrewsbury, U.K. An MZ Hema guard plus 2 x Hema Linear column was used for analysis. Since γ-PGA is insoluble in organic solvents, they cannot be used as an eluent. Hence, 0.2 M $NaNO_3$, 0.01 M NaH_2PO_4 at pH 7 was used as the eluent with a flow rate of 1.0 ml/min at 30°C and a Refractive Index (RI) detector (with differential pressure and light scattering). The data was collected and analysed using Polymer Laboratories "Cirrus" software. Sample preparation involved adding 10 ml of eluent to 20 mg of sample to give a concentration of 2 mg/ml. This was left overnight to dissolve. The solutions were well mixed and filtered through a 0.45 µm PVDF membrane into autosampler vials. GPC system used for this work was calibrated with sodium polyacrylate calibrants obtained from Polymer Laboratories.

3.1.7.5 Crystallinity of γ-PGA

Crystallinity of a polymer influences polymer properties such as hardness, tensile strength, stiffness, solubility and melting point. Hence, it plays an important role when selecting a polymer for an application. A polymer usually contains both crystalline and amorphous phases arranged randomly. A crystalline phase is where the atoms are arranged in a regular

repeating pattern in the structure. In contrast, in an amorphous phase, there is no such order and the arrangement is haphazard.

Crystallinity was assessed at Queen Mary University of London using powder X-Ray Diffraction (XRD) analysis. XRD is an analytical technique where a beam of X-ray is passed through a polymer sample. Regularly arranged atoms (crystalline phase) in the sample reflect the beam constructively and are represented by sharp, narrow diffraction peaks on the XRD spectrum, whereas randomly occurring atoms (amorphous phase) would be represented by very broad peaks. Data from XRD analysis of γ-PGA was collected at room temperature with a Phillips PW1700, 40kV/40mA, CuKα instrument.

3.2 PROBIOTICS

3.2.1 Bacterial strains for probiotic tests

Three probiotic bacteria (*Bifidobacterium longum* NCIMB 8809, *Bifidobacterium breve* NCIMB 8807 and *Lactobacillus paracasei* NCIMB 8835) were used for the tests. All bacteria were obtained from NCIMB. The stock culture was freeze-dried and stored at -20°C. Before use, the cultures were revived aseptically and grown anaerobically on Bifidus Selective Medium Agar (BSM Agar) for *Bifidobacteria* and DeMan Rogosa Sharpe (MRS) agar for *Lactobacillus* at 37°C in jars using an atmosphere generation system (Oxoid Anaerogen™) and indicator strip.

3.2.2 Growth media

Trypticase-Phytone-Yeast extract (TPY) and BSM were used for growth and enumeration of *Bifidobacteria* under study (Nualkaekul *et al.*, 2011). TPY can be used for enumeration of *Bifidobacteria*, but it is important to note that it is not a selective medium. TPY broth and agar were prepared in the lab from their constituent ingredients. The composition for TPY medium is given in **Table 3.5**.

Table 3.5: Composition of TPY medium

Chemical	Amount	Source
Lag phase tryptone	10g/l	Lab M
Neutralised soya peptone	5g/l	Oxoid
Glucose	5g/l	Thornton & Ross
Tween 80	1ml/l	Acros Organic
Yeast extract	2.5g/l	Lab M
Cystein hydrochloride	0.5 g/l	Sigma Aldrich
Dipotassium phosphate, K_2HPO_4	2g/l	Sigma Aldrich
Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$	0.5g/l	May & Baker
Zinc sulphate heptahydrate, $ZnSO_4 \cdot 7H_2O$	0.25g/l	AnalaR
Calcium chloride, $CaCl_2$	0.15g/l	Fisher Chemical
Ferric chloride, $FeCl_3$	Trace	Sigma Aldrich
Agar No. 2 (For TPY agar)	15g/l	Lab M

The final pH of the medium was adjusted to 6.5 ± 0.1 using 3 M NaOH and 3 M HCl. TPY was sterilized at 110ºC and 0.35 BAR for 30 mins to avoid caramelization of glucose.

For tests which required enumeration of *Bifidobacteria* in the probable presence of other bacteria, BSM agar was used. BSM is used for the selective isolation, identification and enumeration of *Bifidobacteria*. This medium contains selective salts that inhibit the growth of moulds, *Lactobacilli*, *Streptococci*, *Enterococci* and other Gram negative bacteria. It also contains a compound that inhibits glycolysis by inactivating glyceraldehyde-3-phosphate dehydrogenase present, which is important in different bacteria and fungi. The presence of

three antibiotics in the medium makes it selective by inhibiting bacterial flora like *Bacilli*, *Enterobacteriaceae* and *Pseudomonas*. In addition, BSM contains an azo compound that *Bifidobacteria* can reduce, which gives the colonies a pink-purple colour.

For *Lactobacillus*, MRS medium was used. BSM agar and MRS broth/agar were purchased and prepared according to the manufacturer's protocol (Sigma-Aldrich, U.K.). The composition of MRS agar/broth has been given in **Tables 3.6** & **3.7** below.

Table 3.6: Composition of MRS agar

Chemical	Amount
Diammonium hydrogen citrate	2 g/l
Dipotassium hydrogen phosphate	2 g/l
D(+)-glucose	20g/l
Magnesium sulphate	0.1 g/l
Manganous sulphate	0.05 g/l
Meat extract	5 g/l
Sodium acetate	5 g/l
Universal peptone	10 g/l
Yeast extract	5 g/l
Agar	12 g/l

The final pH of the medium was 6.5 ± 0.2.

Table 3.7: Composition of MRS broth

Chemical	Amount
Dipotassium hydrogen phosphate	2 g/l
D(+)-glucose	20g/l
Magnesium sulphate heptahydrate	0.2 g/l
Manganous sulphate tetrahydrate	0.05 g/l
Meat extract	8 g/l
Peptone	10 g/l
Sodium acetate trihydrate	5 g/l
Triammonium citrate	2 g/l
Yeast extract	4 g/l

The final pH of the medium was 6.2 ± 0.2

MRS and BSM were prepared using de-ionised water and sterilized at 121ºC and 1.035 BAR for 20 min.

3.2.3 Sterilization of γ-PGA

Sterilization of γ-PGA is an important step before using it for a probiotic application to make sure that the residual cell population in the γ-PGA sample does not interfere with the subsequent results of probiotic tests. There is no reference to sterilization of γ-PGA in the literature. Therefore, several sterilization techniques were assessed before selecting the most appropriate for the future tests.

Method 1: 10% γ-PGA solution was filter sterilized using a 0.45µm filter.

Method 2: The effect of ethanol treatment on γ-PGA was also tested. 95% ethanol was added to γ-PGA. Since γ-PGA is insoluble in ethanol, the ethanol treated γ-PGA was centrifuged at 17000g for 15 minutes. The ethanol (supernatant) was aspirated using a sterile pipette and the excess ethanol was evaporated at 50ºC in an oven to obtain a dry γ-PGA powder. This powder was checked for residual contaminants by serially diluting a solution of γ-PGA in sterile 1/4th strength Ringer solution and plating on TSA plates which were incubated aerobically/anaerobically for 24-48 h at 37˚C.

Method 3: γ-PGA was also treated with different concentrations of H_2O_2 (1%, 3%, or 5%) for 30 minutes. γ-PGA is soluble in H_2O_2. Hence, after treatment, γ-PGA was extracted and isolated using ethanol precipitation according to the description in **section 2.1.4**. After precipitation, γ-PGA was centrifuged at 17000g for 30 minutes to obtain wet pellets of γ-PGA. The pellets were dried in the oven at 50˚C to obtain dry γ-PGA powder and this was checked for contamination as described previously.

Method 4: 10% γ-PGA solution was autoclaved at 121˚C at 1.035 BAR for 20 mins and at 110˚C at 0.35 BAR for 30 mins. The autoclaved solutions were checked for contamination as described previously.

FT-IR analysis was performed on γ-PGA samples sterilized by H_2O_2 treatment and autoclaving to identify any change in the identifiable bonds within γ-PGA due to the sterilization process.

3.2.4 Scanning Electron Microscopy (SEM) analysis

SEM analysis was performed to determine the surface structure of freeze dried cells protected with γ-PGA. Samples were prepared by freeze drying and grinding the γ-PGA-protected and unprotected cells to obtain a fine dry powder. Samples were then coated with gold using a sputter coater. Analysis was done using an SEM (Zeiss EVO50, UK) and photographs were analysed using the Smart SEM software.

3.2.5 γ-PGA as cryoprotectant

Cryoprotectant tests were first performed with γ-PGA that was sterilized by autoclaving at 110˚C and 0.35 BAR for 30 mins (henceforth mentioned as γ-PGA[S]). For these tests, TPY medium was used for growth and enumeration of cells, since the presence of residual bacteria in γ-PGA[S] had been eliminated by sterilization. Hence, a selective media for enumeration was not necessary. To ascertain whether autoclaving had any effect on the cryoprotective ability of γ-PGA, cryoprotectant tests were repeated with untreated γ-PGA (γ-PGA[U]). However, for these tests, it was necessary to use a selective and differential media (BSM) for enumeration of cells protected with γ-PGA[U], because of the probable presence of some

residual bacteria in the γ-PGA sample. To compare results of cryoprotectant tests with γ-PGA[S] and γ-PGA[U], it was necessary to confirm that the growth of *Bifidobacteria* on TPY and BSM was comparable. Therefore, *B. breve* and *B. longum* were grown in TPY broth (22 h for *B. breve* and 16 h for *B. longum*) at 37 °C anaerobically. After incubation, cells were enumerated on TPY agar and BSM agar and viable cell counts on both media were compared.

To protect cells with cryoprotectant before freeze drying, *Bifidobacteria* were inoculated in TPY broth (22 h for *B. breve* and 16 h for *B. longum*) and *Lactobacillus paracasei* was inoculated in MRS broth (for 48 h) at 37°C anaerobically (as described previously). After incubation, viable counts were taken on TPY/BSM agar (*Bifidobacteria*) and MRS agar (*Lactobacillus*) to determine number of viable cells before freeze drying. The cultures were then centrifuged and washed with Phosphate Buffered Saline (PBS) to obtain cell pellets. Cells were then mixed thoroughly in 10 ml solutions of 20% γ-PGA, 10% γ-PGA, 5% γ-PGA and 10% sucrose. For cells without a cryoprotectant, 10 ml of water was added. The suspensions were incubated at room temperature for 1 h and then frozen at -80°C for 24 h. The frozen cultures were then freeze dried (Edwards Freeze Dryer, UK) at -40˚C and 5 MBAR pressure for 48 h. After freeze drying, 10 ml of PBS was added to all the cells and viability was measured to determine number of viable cells after freeze drying. After appropriate dilution, samples were plated on suitable media and incubated at 37˚C anaerobically for 48 h, as described previously. All tests were done in triplicate. These tests were repeated with γ-PGA[U] (for *B. breve* and *B. longum*).

3.2.6 Protection in fruit juice

It is important to develop non-dairy foodstuffs for delivering probiotics. Commercially available orange juice (Tropicana orange juice, Pure Premium Smooth No Bits) and

pomegranate juice (POM Wonderful 100% Pomegranate juice) were purchased from Waitrose, Wolverhampton, UK. Fresh samples of these fruit juices were requested. These juices were selected because they did not contain any preservatives and they have nutritional value. Bacteria were grown anaerobically (as described previously) in TPY broth (22 h for *B. breve* and 16 h for *B. longum*) at 37 °C. The culture was then centrifuged and washed with PBS to obtain cell pellets. Pellets were then mixed thoroughly in a 10% γ-PGA[S] solution. This mixture was incubated at room temperature and frozen at -80 °C and freeze dried to obtain a dry powder containing cells protected with γ-PGA. 1 ml PBS was added to the dry powder and this solution was transferred to 40 ml of orange juice. The final concentration of γ-PGA in fruit juice was ~ 2.5 % (w/v). For tests with unprotected cells, cells were inoculated in TPY broth using the aforementioned conditions. Cell pellets were obtained after centrifugation and washing with PBS. 1 ml of PBS was added to the cells. This solution was added to orange juice. Viability was measured at day 0, 2, 4, 6, 8, 11, 13, 20, 28 and 39 for unprotected and γ-PGA-protected cells on BSM agar. After appropriate dilution, samples were plated on BSM agar and incubated anaerobically (as described previously) at 37˚C for 48 h.

3.2.7 Organic acid concentration in fruit juice

It was important to assess the change in organic acid composition of fruit juice when unprotected and γ-PGA-protected cells were added to it to determine any alteration in its nutritional and organoleptic properties. Organic acid analysis was performed using HPLC with an HP series 1100 HPLC instrument at the University of Reading, U.K. A Prevail Organic Acid 5µm column with a UV detector was used for citric acid, malic acid, lactic acid, acetic acid and ascorbic acid. 25 mM KH_2PO_4 (pH 2.5 adjusted with phosphoric acid) was used as the eluent. Before loading, the samples were filtered using 0.45 µm filters and

were diluted 10 fold. Standard curves were prepared using known concentrations of chemicals to be analysed (**Fig 3.2**).

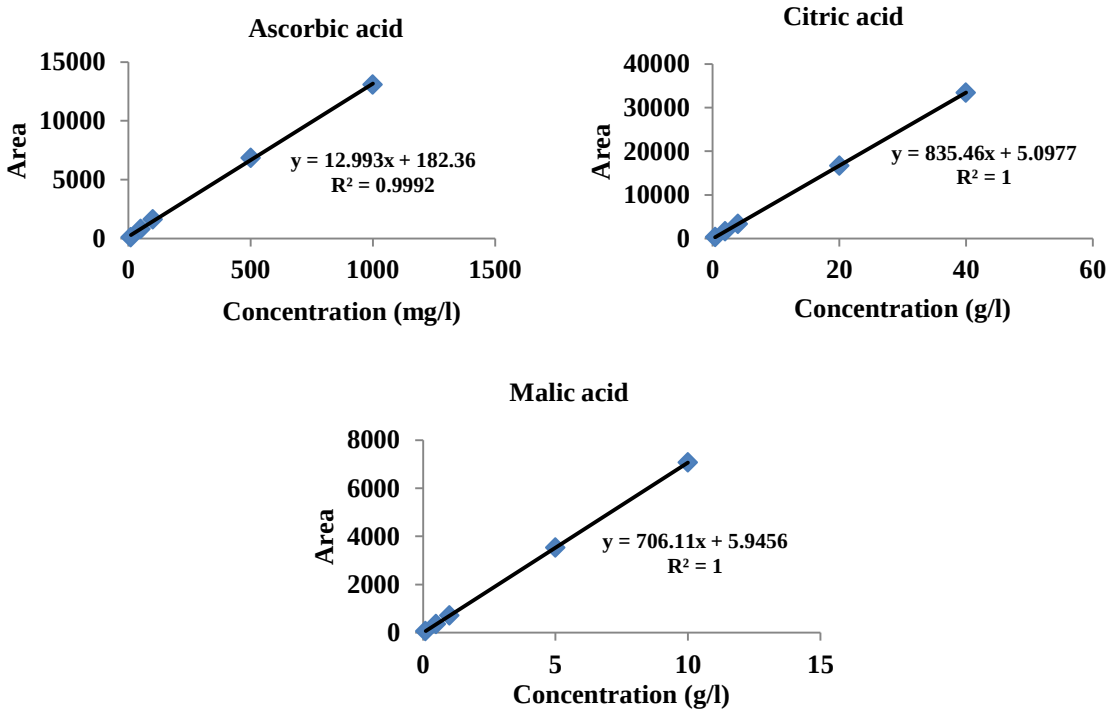


Fig 3.2: Ascorbic acid, citric acid and malic acid standard curves for determination of change in organic content in orange and pomegranate juice on addition of unprotected probiotic cells and γ-PGA-protected probiotic cells using HPLC. Standard error bars are too small to be seen.

3.2.8 Protection in simulated gastric juice

There is a heavy loss in the viability of probiotic bacteria when they pass through the stomach, which is a region of high acidity. The time required for food to transit through the stomach varies by individual, ranging from 0.5 to 4.5 hours (Hsieh *et al.*, 2009). Hence, the protective effect of 2.5% γ-PGA was tested on viability of the 2 *Bifidobacteria* strains when stored in simulated gastric fluid for 4 h. Simulated gastric fluid contained 2 g/l NaCl, 3.2 g/l pepsin powder and 20 ml of 1 M HCl (British Pharmacopoeia Commission, 1993). The components were diluted in 900 ml deionised water and after pH adjustment (pH 2.0), the volume was topped up to 1 L. pH was adjusted using 1 M HCl or 1 M NaOH. This model

solution was sterilized by passage through a sterile 0.2 µm syringe filter. Tests were carried out in an Electrotek Anaerobic Workstation where a microaerophilic environment was maintained (10% CO_2 and 5% O_2). This would also test the effect of γ-PGA on the viability of bacteria when exposed to some oxygen just before ingestion. The aforementioned protocol was followed to protect cells with γ-PGA. Viability was measured at 0, 1, 2, 3 and 4 h for γ-PGA-protected and unprotected cells on BSM agar as described previously.

3.3 STATISTICAL ANALYSES

All results were analysed using Microsoft Excel 2010 and GraphPad Prism 5. Two-factor Anova and student's T test were used to compare data. The Bonferroni multiple comparison test was used for non-parametric analysis of data to determine the difference between individual groups in a data set. P value ≤ 0.05 was considered to be statistically significant.

4. RESULTS - BACTERIAL γ-PGA PRODUCTION

IN SHAKE FLASKS

4.1 Introduction

This section describes the production of γ-PGA using eight different strains of *Bacillus* in GS and E media. Of these bacteria, *B. subtilis* natto and *B. licheniformis* 9945a have been investigated for γ-PGA production previously (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007; Shih and Van, 2001). However, the remaining six bacteria used in this study have not previously been used for this purpose. Hence, this study would contribute new bacteria for γ-PGA production. Samples were taken at regular intervals during the shake flask cultures to assess growth of bacteria producing γ-PGA. In addition, nutrient consumption analysis was performed to assess the bacterial utilization of nutrients in different growth media to identify which medium is best suited for growth and γ-PGA production for each bacterium. After production of γ-PGA in shake flasks, yield of γ-PGA obtained by bacteria in different growth media was evaluated and the product was identified using FT-IR. On identifying the polymer as γ-PGA, the form, crystallinity and molecular weight was assessed using ICP-AES, XRD and aqueous based GPC respectively. Characterization of γ-PGA with respect to these properties is important to produce a more consistent and a better quality product. Identifying different properties of γ-PGA is also crucial because they determine for which application γ-PGA could be used. It was found that the yield of γ-PGA and other properties, such as molecular weight, crystallinity and form (acid/salt) can be different for each bacterium in each medium.

4.2 Growth and nutrient utilization for *Bacillus* strains

Eight different bacteria were grown in GS and E media at 37˚C and 150 rpm for 96 h in shake flasks to investigate the production of γ-PGA. Samples were taken aseptically at regular intervals (6, 24, 48, 72 and 96 h) to determine growth and assess how nutrients in both media were utilized by the cells. It is important to ascertain how nutrient utilization and growth

change with different bacteria and media to get more insight into appropriate medium for bacterial growth and γ-PGA production. It is also important to identify a relationship between growth and utilization of a nutrient, if any, for each bacterial strain. Growth was measured using the Miles & Misra plate count technique. Nutrient consumption analysis was performed using HPLC. L-glutamic acid and sucrose consumption was evaluated for bacterial culture grown in GS medium, whereas L-glutamic acid and glycerol consumption was assessed for those grown in medium E.

4.2.1 *B. subtilis* 23856

In GS medium, a rapid increase in cell count (from 4.96 log CFU/ml to 7.8 log CFU/ml. See **Fig 4.1a**) was observed in the first 24 h. The bacterial cell count reached maximum by 24 h, after which stationary phase commenced. A reduction in cell count was seen after 72 h. *B. subtilis* 23856 also utilized a considerable amount (37.76 g/l of the 50 g/l provided) of sucrose in the first 24 h of growth. Eventually, bacteria consumed 12.94 g/l of the 20 g/l of L-glutamic acid provided and 45.33 g/l of sucrose by 96 h. The concentration of sucrose in the medium did not change significantly once cells entered death phase (after 72 h).

In medium E, an increase in the viable cell count for *B. subtilis* 23856 (from 5.40 log CFU/ml to 7.41 log CFU/ml. See **Fig 4.1b**) in the first 24 h of growth was accompanied by consumption of 27.23 g/l of glycerol. The maximum cell count (7.95 log CFU/ml) was achieved by 72 h of growth in medium E. Bacteria consumed approximately half (39.91 g/l) of the provided glycerol by 96 h. Some glycerol was also consumed after the cell count started decreasing after 72 h (i.e. during death phase). Also, only 4.89 g/l of L-glutamic acid was utilized by the cells at the end of 96 h.

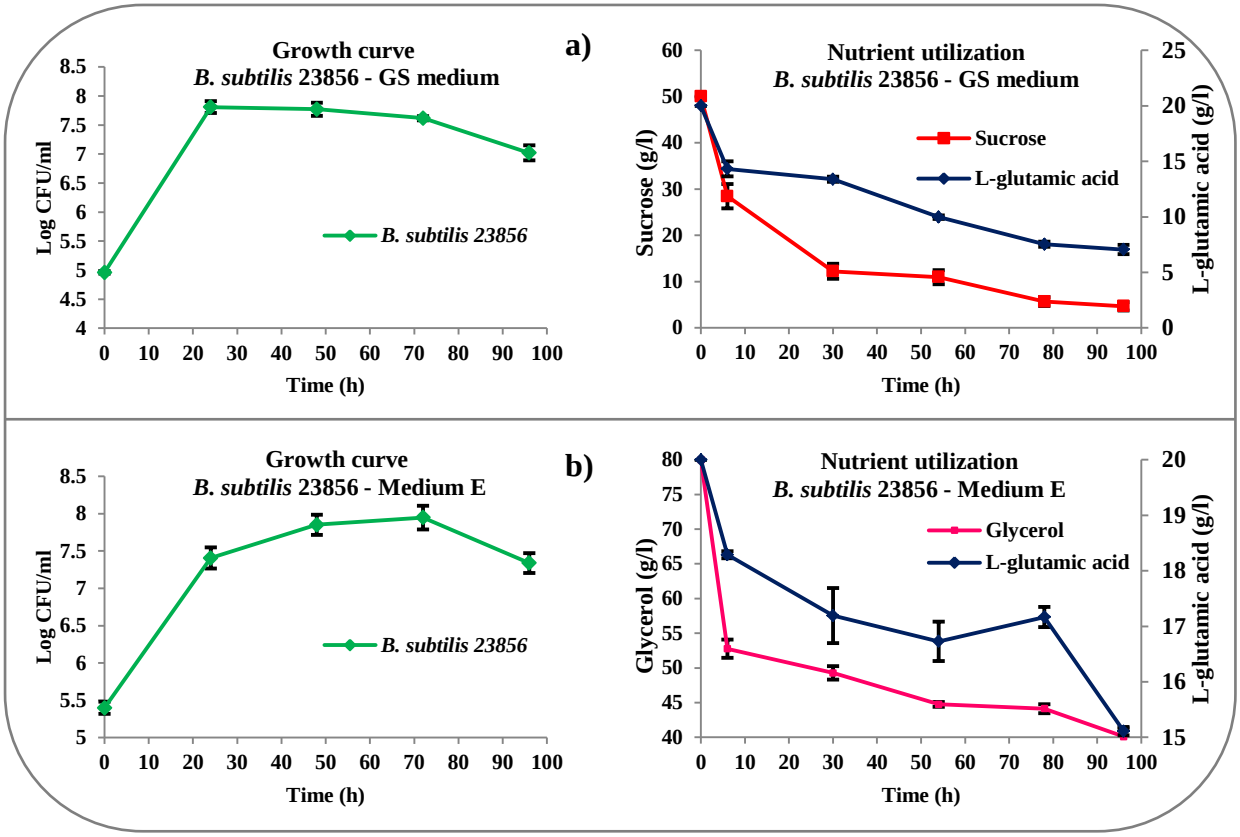


Fig 4.1: Growth and nutrient utilization for *B. subtilis* 23856 when grown in a) GS medium & b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.2 *B. subtilis* 23857

When *B. subtilis* 23857 was grown in GS medium, the cell count increased (from 4.96 log CFU/ml to 7.93 log CFU/ml) in the first 24 h, which is when the maximum cell count was attained (**Fig 4.2a**). A rapid decrease in the sucrose and L-glutamic acid concentration in first 24 h of growth was observed. Eventually, cells utilized 44.09 g/l of the 50 g/l of sucrose provided and 11.70 g/l of the 20 g/l of L-glutamic acid provided by 96 h.

In medium E, the maximum cell count was attained by 48 h (7.72 log CFU/ml), although cell counts at 24 h and 72 h were not significantly different ($P > 0.05$. See **Fig 4.2b**). Rapid cell growth in the first 24 h was accompanied by a reduction in glycerol (from 80 g/l to 47.78 g/l) and L-glutamic acid concentration (from 20 g/l to 16.64 g/l) in the medium. Eventually cells

consumed 43.34 g/l of glycerol by 96 h, whilst only 5.94 g/l of L-glutamic acid was consumed by this time. No drastic loss in cell viability was seen after 72 h.

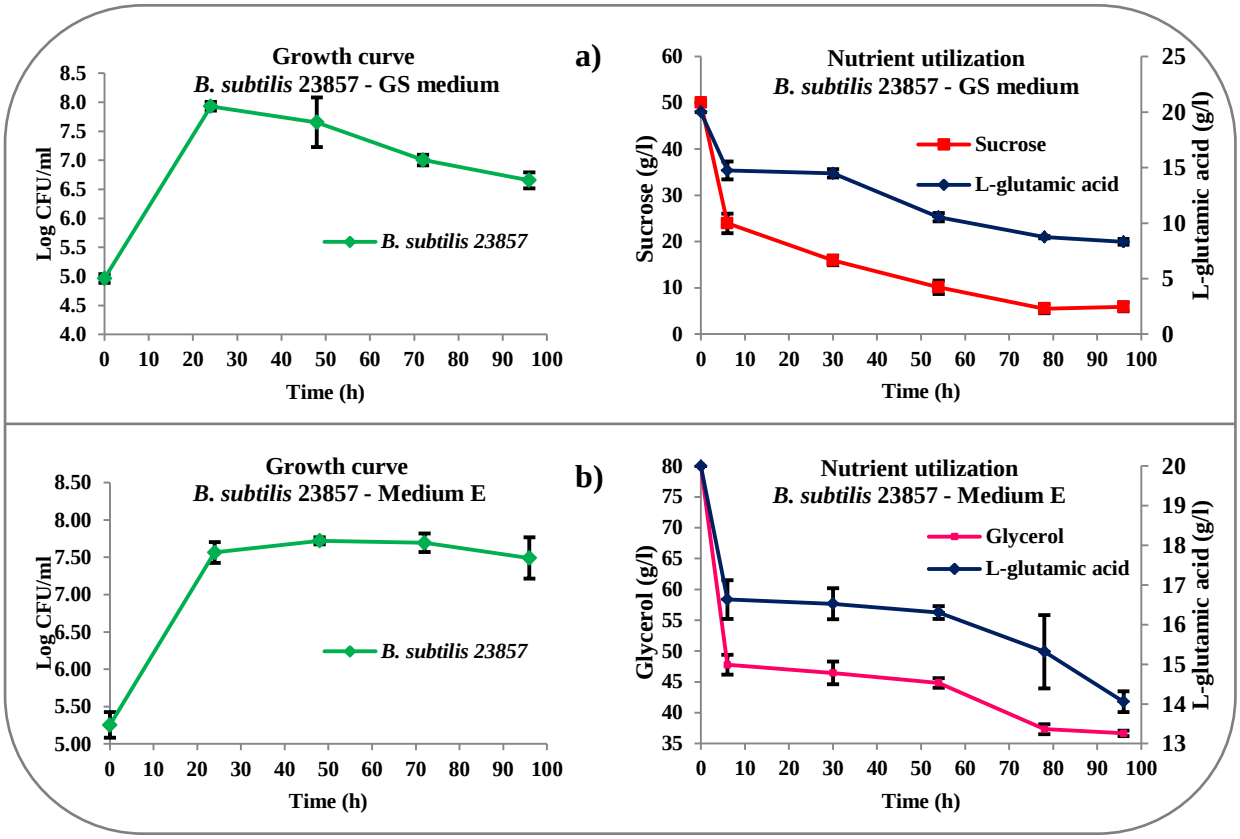


Fig 4.2: Growth and nutrient utilization for *B. subtilis* 23857 when grown in a) GS medium and b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.3 *B. subtilis* 23858

Bacillus subtilis 23858 reached a maximum cell count of 9.58 log CFU/ml after 72 h of growth in GS medium (**Fig 4.3a**). A steady decrease in sucrose concentration was seen over 96 h, with 45.38 g/l of sucrose being consumed by the cells at the end of the culture. The L-glutamic acid concentration decreased to 3.55 g/l after 96 h, indicating that cells utilized most of the provided nutrients. Although L-glutamic acid concentration increased slightly after 72 h, it was not significantly differently from the concentration at 72 h ($P > 0.05$).

In medium E, the cell count of *B. subtilis* 23858 did not reach as high as that in GS medium (**Fig 4.3b**). A rapid increase in cell growth was seen in the first 24 h, when viable cell count increased from 5.10 to 7.60 log CFU/ml. The maximum cell count was achieved after 72 h (7.91 log CFU/ml) and 40.20 g/l of glycerol was consumed by the cells by 96 h. The glycerol concentration was seen to decrease slightly even after cell death had commenced. 5.70 g/l of the 20 g/l of L-glutamic acid provided was utilized by cells at the end of 96 h.

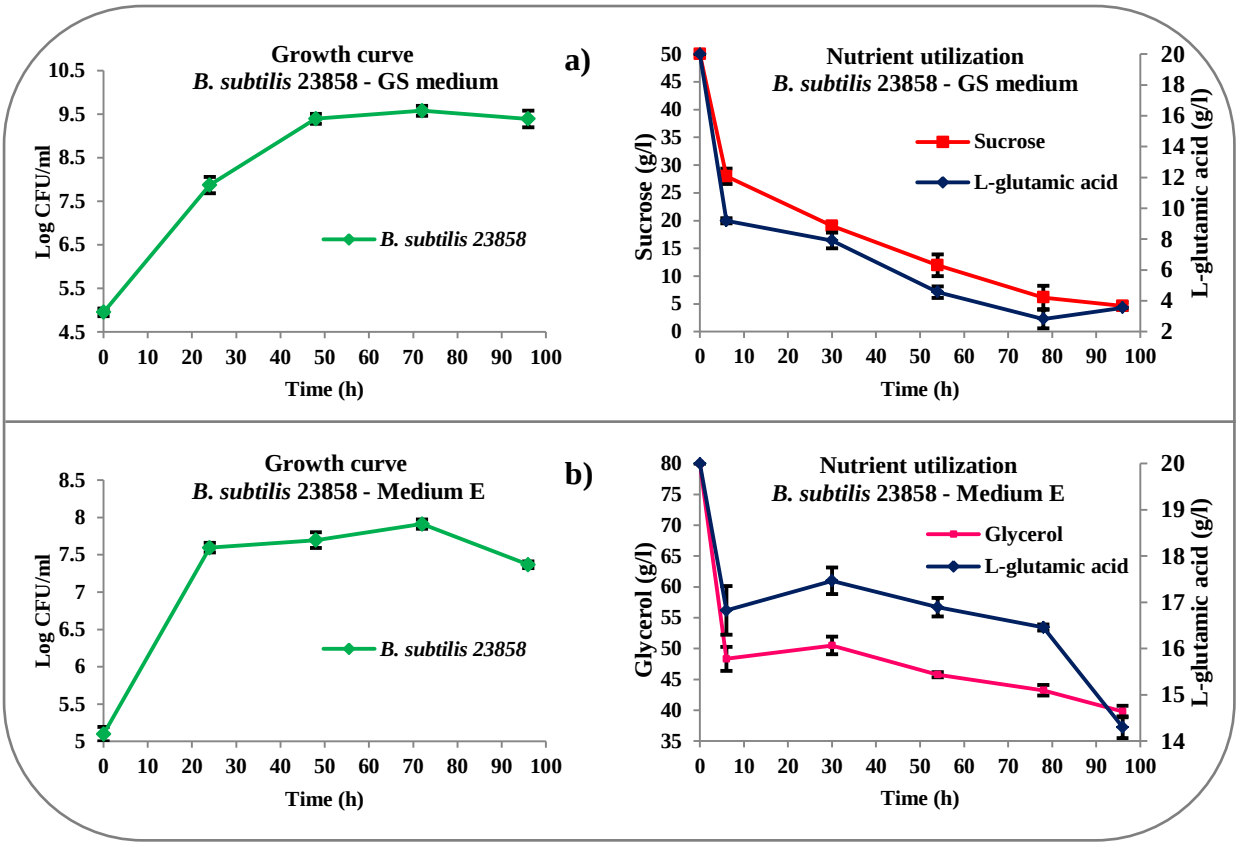


Fig 4.3: Growth and nutrient utilization for *B. subtilis* 23858 when grown in a) GS medium and b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.4 *B. subtilis* 23859

In GS medium, *B. subtilis* 23859 grew to a maximum cell count of 8.76 log CFU/ml (**Fig 4.4a**). Rapid cell growth was seen in the first 24 h of growth (5.08 – 7.43 log CFU/ml), when bacteria consumed 16.82 g/l of sucrose. At the end of 96 h, a total of 42.16 g/l of sucrose was

consumed. With the exception of the growth period between 6 h and 24 h, when only 1.49 g/l of sucrose was consumed, a steady decrease in the sucrose concentration was seen over 96 h. A rapid decrease in the L-glutamic acid concentration (9.76 g/l) was also seen in the first 6 h of growth, after which a steady decrease was observed. At the end of the culture, 13.45 g/l of L-glutamic acid was consumed by the cells.

In medium E, *B. subtilis* 23859 reached a maximum cell count of only 7.55 log CFU/ml after 24 h (**Fig 4.4b**). A loss in viability was seen between 72 and 96 h (from 7.14 to 4.93 log CFU/ml). A total of 50.37 g/l of glycerol and 5.26 g/l of L-glutamic acid were consumed by 96 h. Glycerol and L-glutamic concentration was seen to decrease even after the cell count started decreasing (after 72 h).

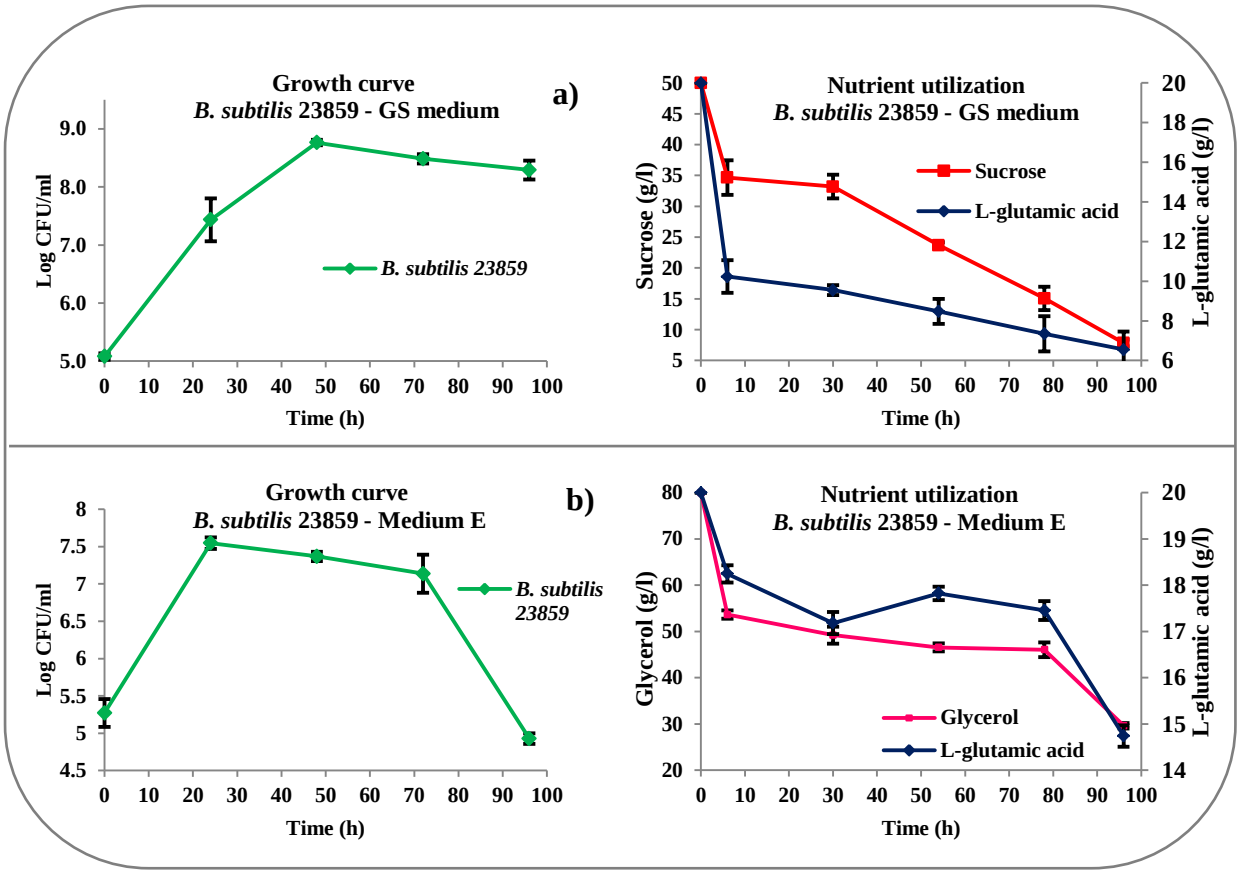


Fig 4.4: Growth and nutrient utilization for *B. subtilis* 23859 when grown in a) GS medium & b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.5 *B. subtilis* natto

For *B. subtilis* natto, a rapid increase in the viable cell count (from 4.99 log CFU/ml to 9.03 log CFU/ml) was observed in the first 24 h of growth, which was accompanied by a rapid utilization of sucrose (**Fig 4.5a**). A loss in cell viability was seen after 72 h, whilst there was no change in the sucrose concentration observed after this time. At the end of 96 h, *B. subtilis* natto utilized almost all (19 g/l) of the 20 g/l of L-glutamic acid provided. The L-glutamic acid concentration rapidly decreased in the first 24 h of cell growth and continued to decrease, even after cell death commenced in the culture.

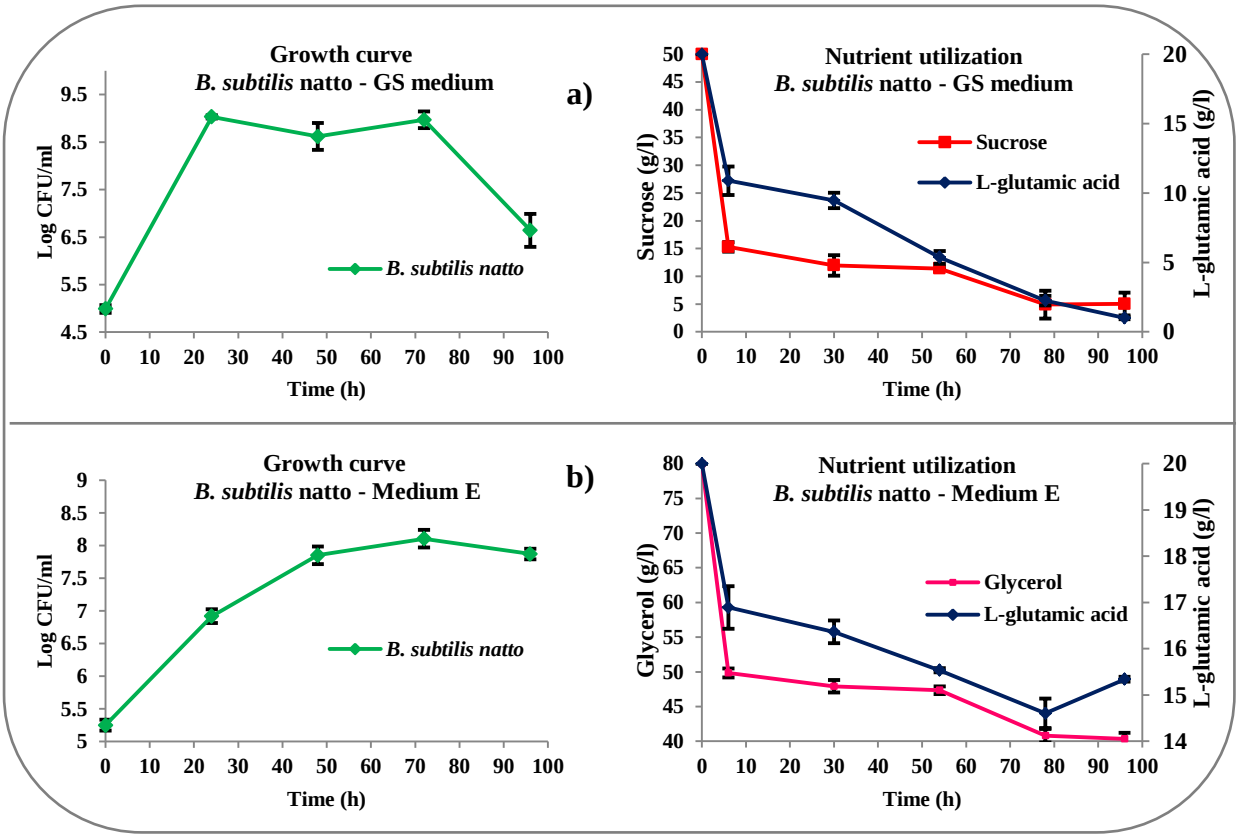


Fig 4.5: Growth and nutrient utilization for *B. subtilis* natto when grown in a) GS medium & b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

In medium E, *B. subtilis* natto reached a maximum cell count (8.11 log CFU/ml) only after 72 h (**Fig 4.5b**). The cells appeared to increase in number gradually and could not grow in medium E as quickly as they did in GS medium. Of the 80 g/l of glycerol provided in medium E, only 39.65 g/l was utilized by *B. subtilis* natto. Approximately 30 g/l of glycerol was consumed in the first 24 h of growth, after which only 9.5 g/l was consumed. Only 4.66 g/l of the provided L-glutamic acid (20 g/l) was consumed after 96 h. Interestingly, the L-glutamic acid concentration at 96 h was significantly higher ($P < 0.05$) than that observed at 72 h. In contrast to observations in GS medium, a rapid reduction in cell count was not seen in medium E after 72 h. This could possibly be due to the fact that nutrients were still available and cells were still in stationary phase at the end of 96 h.

4.2.6 *B. licheniformis* 1525

In GS medium, rapid cell growth of *B. licheniformis* 1525 was observed for the first 24 h, reaching a maximum cell count of 9.02 log CFU/ml after 48 h (**Fig 4.6a**). This was followed by stationary phase, with cell death being seen after 72 h in culture. 47.38 g/l of sucrose was consumed at the end of 96 h, although very little sucrose was consumed after 72 h, when cell viability decreased. A total of 7.84 g/l of L-glutamic acid was consumed by the cells.

In medium E, cells grew to a maximum cell count of 8.57 log CFU/ml by 72 h, after which a reduction in cell viability was seen (**Fig 4.6b**). *B. licheniformis* 1525 utilized 52.51 g/l of glycerol and 6.84 g/l of L-glutamic acid after 96 h. Glycerol and L-glutamic acid concentration was seen to decrease even after cell death commenced in the culture.

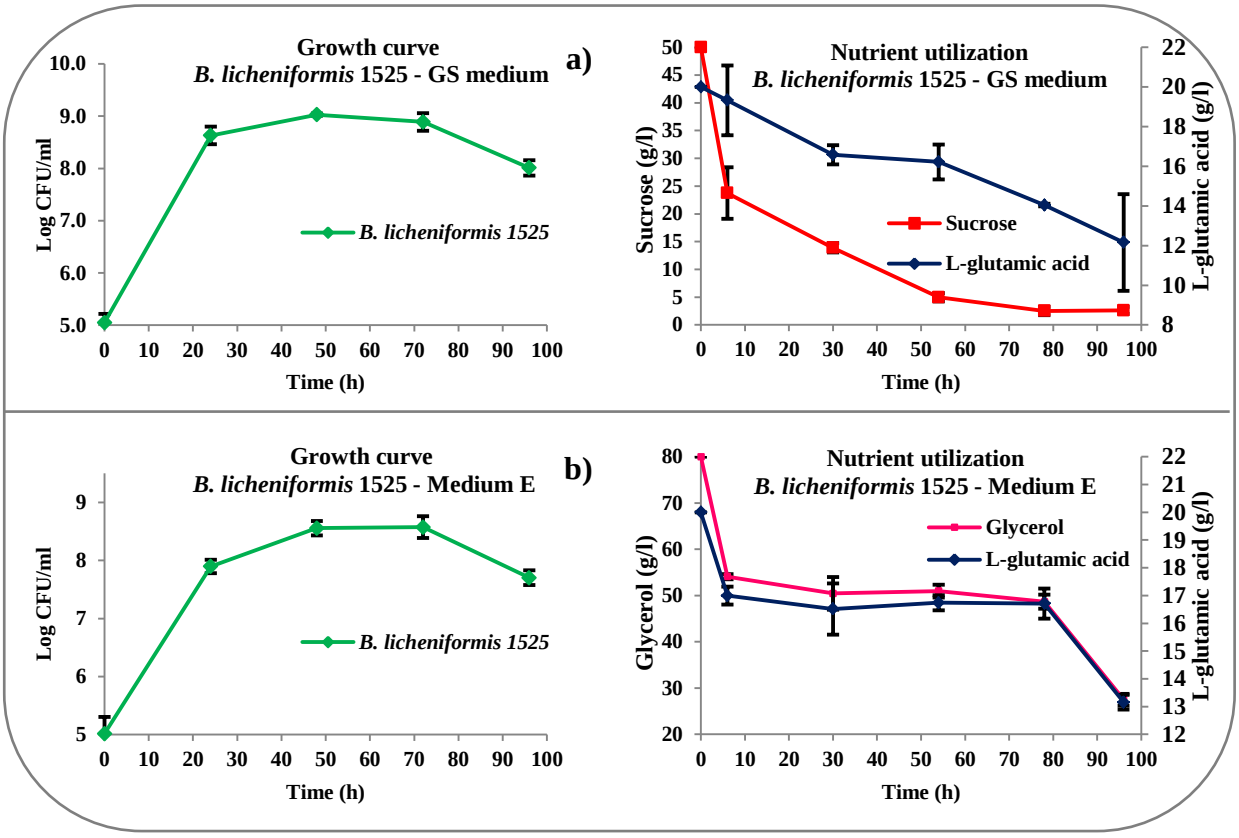


Fig 4.6: Growth and nutrient utilization for *B. licheniformis* 1525 when grown in a) GS medium and b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.7. *B. licheniformis* 6816

In GS medium, *B. licheniformis* 6816 achieved a maximum cell count of 8.95 log CFU/ml after 24 h, after which stationary phase commenced (**Fig 4.7a**). Very little (1.65 g/l) sucrose was consumed after 72 h, when cell death commenced in the culture. After 96 h, cells consumed 48.98 g/l of sucrose and 6.77 g/l of L-glutamic acid.

When cells were grown in medium E, a maximum cell count of 8.54 log CFU/ml was achieved after 48 h (**Fig 4.7b**). Cells were seen to grow rapidly in the first 24 h (increasing from 5.59 to 8.34 log CFU/ml). A drastic loss in viability was seen after 72 h (from 8.42 to 6.59 log CFU/ml). The glycerol concentration was also seen to reduce during this period. At

the end of 96 h, the culture had utilized 54.07 g/l and 8.89 g/l of glycerol and L-glutamic acid provided respectively.

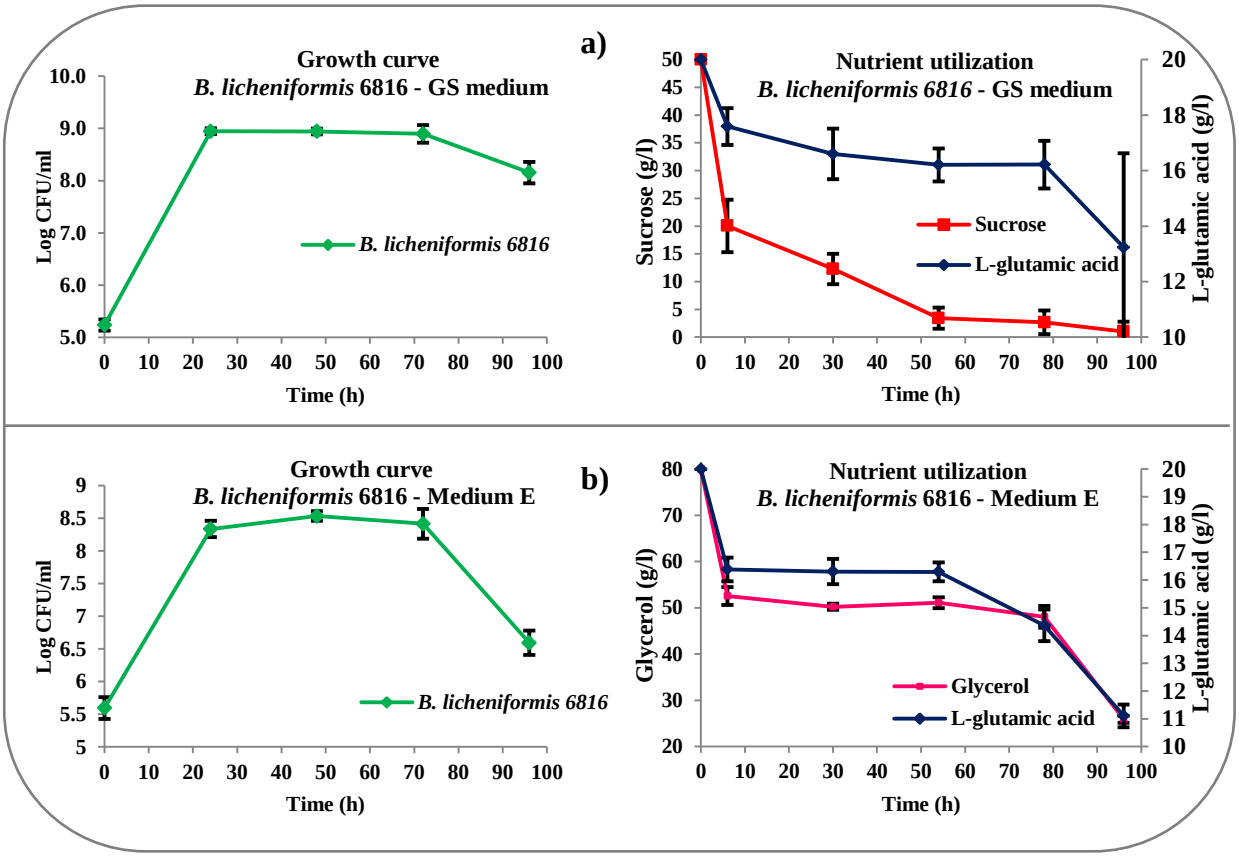


Fig 4.7: Growth and nutrient utilization for *B. licheniformis* 6816 when grown in a) GS medium and b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.2.8. *B. licheniformis* 9945a

When *B. licheniformis* 9945a was grown in GS medium, they reached a maximum cell count of 8.73 log CFU/ml after 48 h (**Fig 4.8a**). After 72 h of growth, cells started losing viability in culture. Cells utilized 43.49 g/l of sucrose after 96 h. Once cell death commenced after 72 h, the sucrose concentration remained constant. Only 3.49 g/l of L-glutamic acid was consumed at the end of 96 h in GS medium. In medium E, cells reached a maximum cell count of 8.41 log CFU/ml (**Fig 4.8b**). Rapid cell growth was seen up to 48 h, after which cell viability started to decline. Cells consumed 56.53 g/l of glycerol in medium E. The concentration of L-

glutamic acid remained constant from 24-72 h. At the end of the culture, 7.21 g/l of L-glutamic acid was consumed.

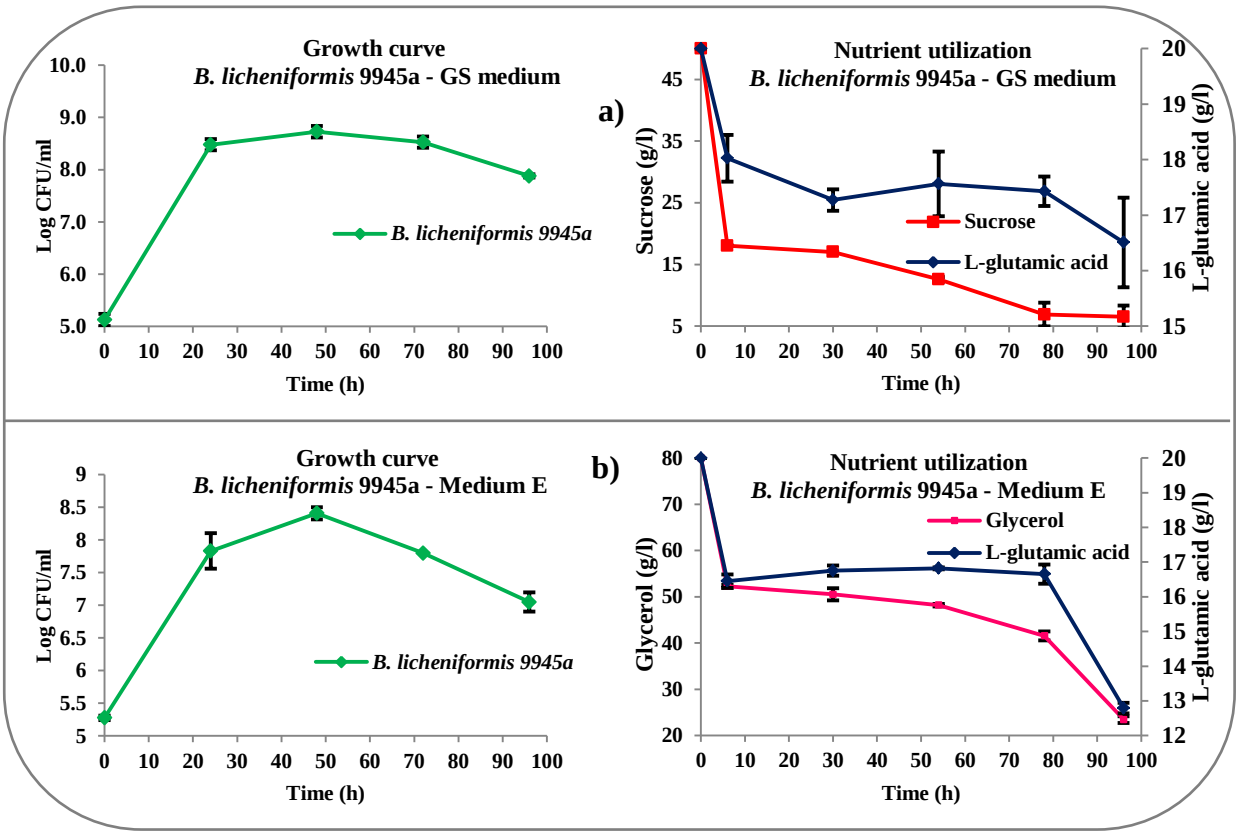


Fig 4.8: Growth and nutrient utilization for *B. licheniformis* 6816 when grown in a) GS medium and b) medium E at 37˚C & 150 rpm for 96 h to evaluate production of γ-PGA. Experiments were conducted in triplicate (n = 3).

4.3 γ-PGA yield

It is essential to have knowledge of how yield of γ-PGA production changes with bacteria and medium of production. This would help identify which bacterial strain amongst those being investigated in this study produces the highest yield of γ-PGA and which medium can be used to give a better yield of γ-PGA for each bacterial strain. After 96 h of shake flask culture, polymers produced by eight bacteria grown in GS and E medium were purified and recovered as dry powders, which were then weighed to calculate yield. **Fig 4.9** represents the γ-PGA yields produced by the eight bacteria in GS and E media. The yields of γ-PGA

produced by *B. subtilis* 23856, *B. subtilis* 23858, *B. licheniformis* 9945a and *B. licheniformis* 6816 in medium GS and E were comparable and were not significantly different ($P \geq 0.05$). Only *B. subtilis* natto produced more γ-PGA in GS medium than in medium E ($P < 0.05$). In fact, *B. subtilis* natto produced the lowest yield of γ-PGA in medium E (5.7 g/l, $P < 0.05$). Of the other bacteria under study, *B. subtilis* 23857, *B. subtilis* 23859 and *B. licheniformis* 1525 produced significantly more γ-PGA in medium E than in GS medium ($P < 0.05$). In GS medium, the lowest yield of γ-PGA was produced by *B. licheniformis* 6816 (13.3 g/l), whereas *B. subtilis* natto produced the highest yield of γ-PGA (17.77 g/l, $P < 0.05$). In medium E, *B. licheniformis* 1525 produced the highest yield of γ-PGA (22.3 g/l, $P < 0.05$).

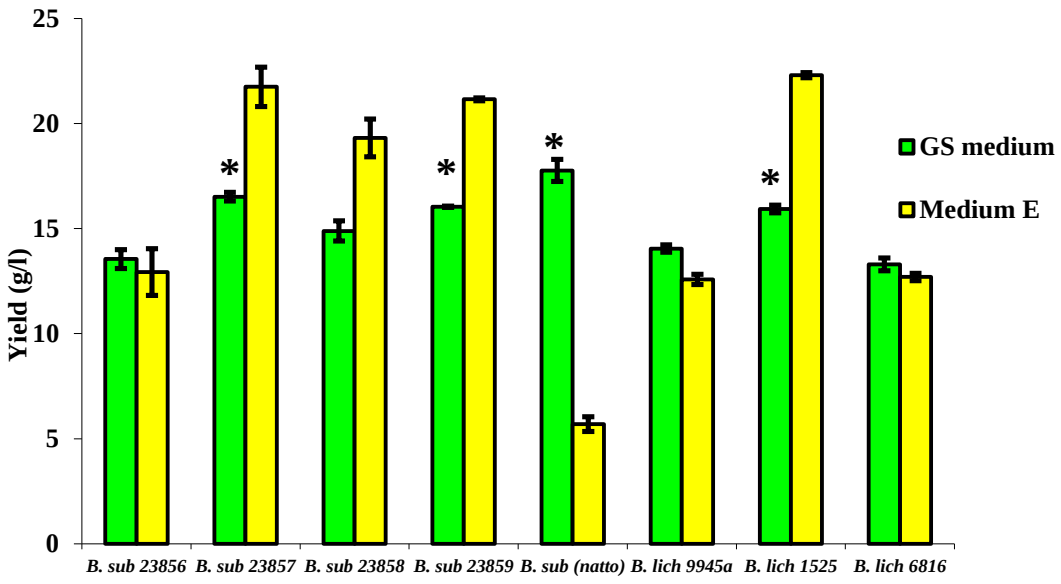


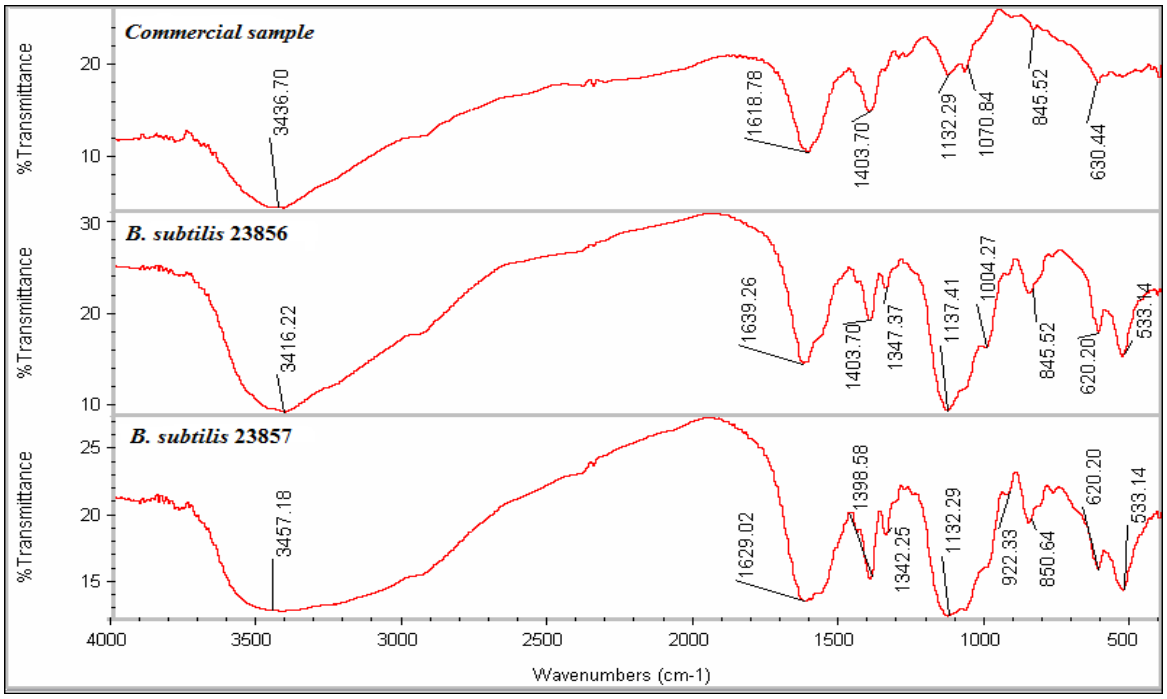
Fig 4.9: Comparison of yields of γ-PGA (g/l) produced by different *Bacillus* strains grown in GS and E medium in flasks and incubated at 37˚C & 150 rpm for 96 h. "*" indicates that the yield of γ-PGA produced by bacteria in GS medium was significantly different ($P < 0.05$) from that in medium E. Experiments were conducted in triplicate.

4.4 Identification of γ-PGA

After 96 h, the polymer produced was recovered in the form of a dry powder. It was observed that cells grown in GS medium produced a white powder. In medium E, cells produced a brownish product. It was important to identify the produced polymer as γ-PGA and hence, FT-IR spectroscopy was performed. The spectra of the polymer produced by eight bacterial strains in both media were also compared to the spectra of a commercially available γ-PGA sample.

The polymer produced by all bacteria was identified as γ-PGA using FT-IR (see **Figs 4.10 & 4.11**). Each spectrum shown is a mean of 3 spectra. FT-IR spectra for γ-PGA obtained by the strains under investigation were similar and they compared well to that of a commercially available γ-PGA sample. The infrared spectra of γ-PGA samples showed characteristic strong amide absorption at ~1618 cm^{-1}, carbonyl absorption at 1395–1454 cm^{-1}, and strong hydroxyl absorption at 3400–3460 cm^{-1}. The absorption peak at 3400–3460 cm^{-1} is characteristic of OH stretching from the bound hydroxyl group and adsorbed water molecules. The absorption peaks around 1600–1640 cm^{-1} and 1390–1410 cm^{-1} are characteristic of amide groups and C=O groups respectively. The strong absorption peaks observed in the range from 1132 cm^{-1} to 1138 cm^{-1} and ~ 620 cm^{-1} are characteristic of C–N groups and N-H, oop bending respectively. γ-PGA produced from all *Bacillus* strains in both media showed the presence of all of these characteristic peaks.

a)



b)

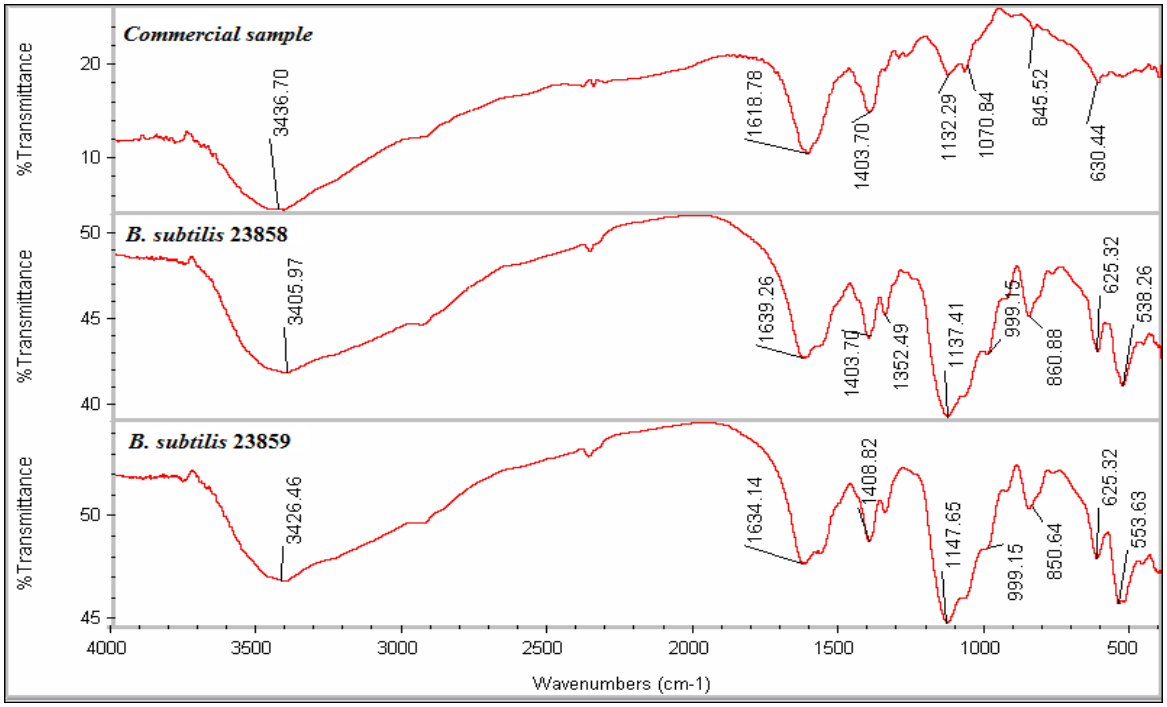
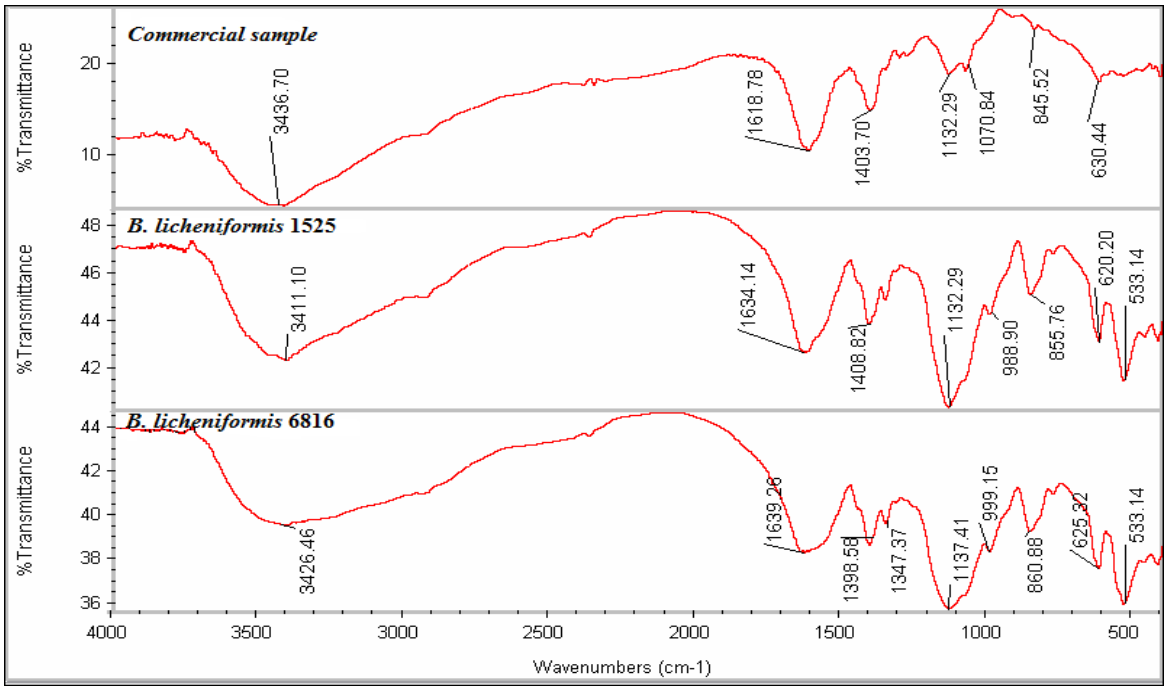


Fig 4.10: FT-IR spectra for γ-PGA produced by a) *B. subtilis* 23856 & 23857 and b) *B. subtilis* 23858 & 23859 in GS medium compared to that of a commercial γ-PGA sample. γ-PGA was produced using eight different bacteria using two media in flasks at 37˚C & 150 rpm for 96 h. Experiments were conducted in triplicate. Each FT-IR spectrum is a mean of 3 spectra.

Fig 4.10 continued overleaf

c)



d)

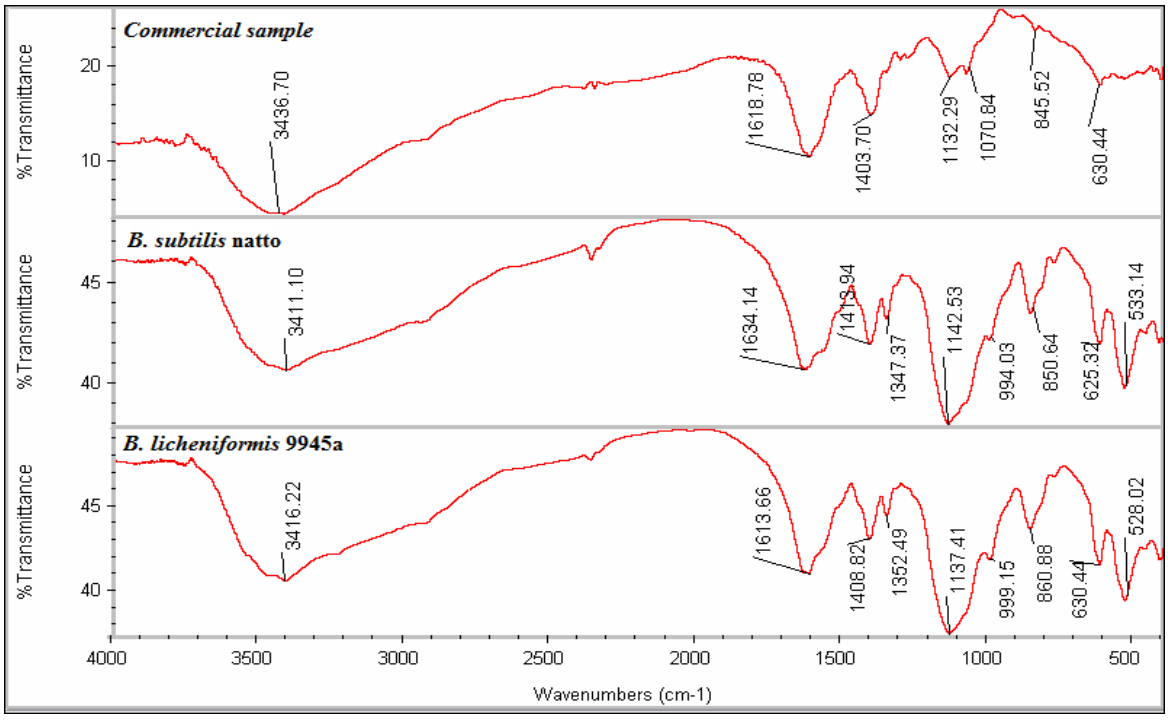


Fig 4.10 (contd): FT-IR spectra for γ-PGA produced by c) *B. licheniformis* 6816 & 1525 and d) *B. subtilis* natto & *B. licheniformis* 9945a in GS medium compared to that of a commercial γ-PGA sample. γ-PGA was produced using eight different bacteria using two media in flasks at 37˚C & 150 rpm for 96 h. Experiments were conducted in triplicate. Each FT-IR spectrum is a mean of 3 spectra.

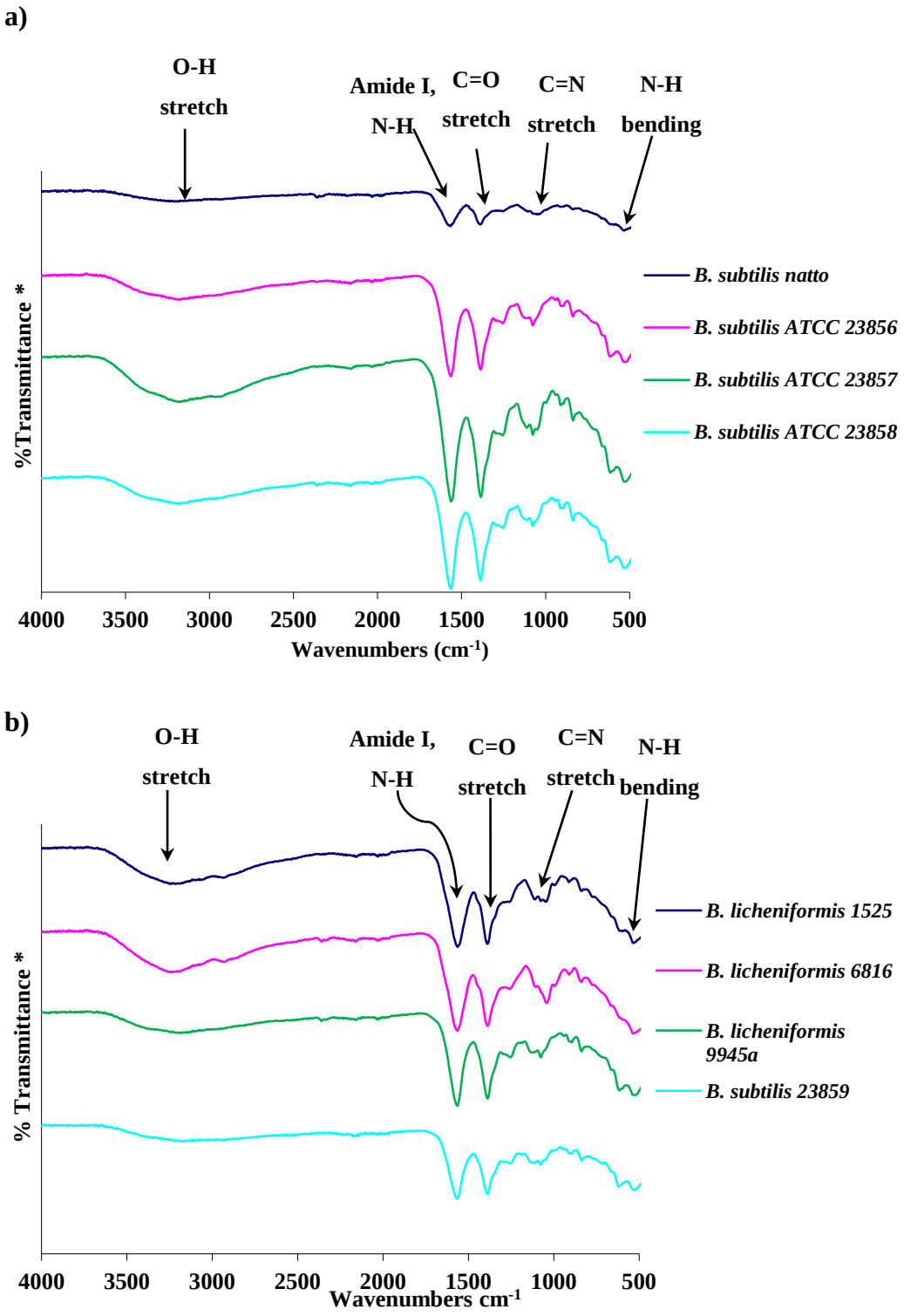


Fig 4.11: FT-IR spectra for γ-PGA produced by a) *B. subtilis* natto, *B. subtilis* 23856, *B. subtilis* 23857 & *B. subtilis* 23858 and b) *B. licheniformis* 1525, *B. licheniformis* 6816, *B. licheniformis* 9945a & *B. subtilis* 23859 in medium E. γ-PGA was produced using eight different bacteria using two media in flasks at 37˚C & 150 rpm for 96 h. Experiments were conducted in triplicate. Each FT-IR spectrum is a mean of 3 spectra.

***Note that % transmittance is dimensionless because these are a composite of 4 individual spectra.**

4.5 Elemental analysis

Before using γ-PGA for any application, it is important to have knowledge of the form of the polymer for a better quality and a more consistent product. Moreover, assessing the form of γ-PGA is important since the percentage salt composition of the polymer would affect its solubility and would in turn affect the application for which it is used. Elemental analysis of γ-PGA produced by different bacteria in two media will also throw light on the factors affecting the form of γ-PGA.

On identifying the polymer as γ-PGA using FT-IR, elemental analysis was performed using ICP-AES to identify whether the salt or the acid form of γ-PGA was produced in both the media (**Table 4.1**). On conclusion of the analysis, it was seen that the majority of the γ-PGA obtained by all bacteria grown in GS medium was the sodium salt of γ-PGA (Na-γ-PGA), with the remainder being either the P, Mg and K salt forms. *B. licheniformis* 1525 produced the highest percentage of Na-γ-PGA (97.39%). None of the strains produced the acid form of γ-PGA (H^+-γ-PGA) in GS medium. In contrast, bacteria grown in medium E produced a considerable amount of H^+-γ-PGA (37-58%) along with Na-γ-PGA. The presence of Na-γ-PGA was possibly because the pH of medium E was adjusted using 3 M NaOH. *B. licheniformis* 6816 produced the highest percentage of H^+-γ-PGA (57.97%) amongst all strains under study.

Table 4.1: Proportions of Na-γ-PGA and H^+-γ-PGA obtained in a) GS medium and b) Medium E. Analysis was performed using ICP-AES (SPECTRO CIROS^{CCD}). Results are expressed as γ-PGA in its Na-salt form and acid (H^+) form. Experiments were conducted in triplicate (n = 3).

a)

Bacteria	% Na-γ-PGA (w/w)
***B. subtilis* 23856**	86.90 ± 3.16
***B. subtilis* 23857**	87.55 ± 0.70
***B. subtilis* 23858**	80.26 ± 4.76
***B. subtilis* 23859**	82.41 ± 0.74
***B. subtilis* natto**	85.38 ± 0.12
***B. licheniformis* 1525**	97.39 ± 2.45
***B. licheniformis* 6816**	88.37 ± 0.25
***B. licheniformis* 9945a**	90.31 ± 3.53

b)

Bacteria	% Na-γ-PGA (w/w)	% H^+-γ-PGA (w/w)
***B. subtilis* 23856**	59.90 ± 1.23	37.76 ± 0.68
***B. subtilis* 23857**	52.68 ± 0.47	44.98 ± 2.63
***B. subtilis* 23858**	56.00 ± 3.61	41.91 ± 1.43
***B. subtilis* 23859**	56.29 ± 0.22	41.73 ± 1.11
***B. subtilis* natto**	52.31 ± 1.10	44.56 ± 4.60
***B. licheniformis* 1525**	50.33 ± 2.96	48.09 ± 0.13
***B. licheniformis* 6816**	38.84 ± 3.33	57.97 ± 3.18
***B. licheniformis* 9945a**	64.47 ± 0.58	32.89 ± 0.19

4.6 Crystallinity

For the same reasons we needed to analyse the form of γ-PGA produced, it is crucial to assess whether the γ-PGA is crystalline or amorphous, before testing it for any application. Crystallinity of a polymer influences polymer properties such as hardness, tensile strength,

stiffness, solubility and melting point. Crystallinity of γ-PGA produced by eight bacteria in two media in this study was analysed using XRD spectroscopy.

The XRD spectra of γ-PGA produced by the strains grown in GS and E media are presented in **Fig 4.12 & 4.13**. Each XRD spectrum is a mean of 3 spectra. Presence of sharp and narrow diffraction peaks was observed in the XRD spectra for γ-PGA obtained from bacteria grown in GS medium (**Fig 4.12**), thus indicating that all strains produced crystalline γ-PGA in GS medium.

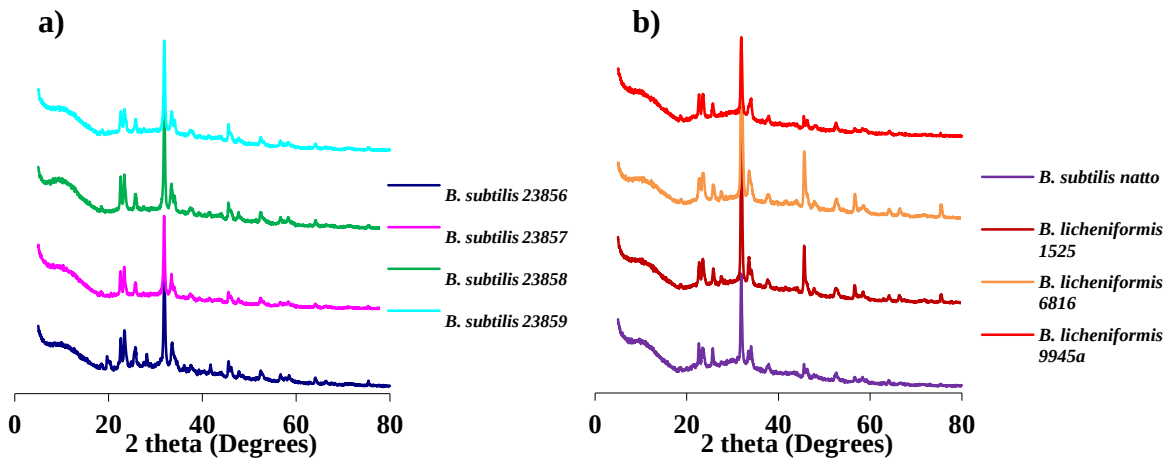


Fig 4.12: Crystallinity of γ-PGA produced by a) *B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859 and b) *B. subtilis* natto, *B. licheniformis* 1525, *B. licheniformis* 6816 and *B. licheniformis* 9945a in GS medium. Crystallinity was assessed by performing XRD spectroscopy (Phillips PW1700). Experiments were conducted in triplicate. Each XRD spectrum is a mean of 3 spectra.

In contrast to the spectra obtained for γ-PGA produced in GS medium, broad peaks were seen to be present in the spectra obtained for γ-PGA produced in medium E (**Fig 4.13**), thus indicating that all bacteria produced amorphous γ-PGA when grown in this medium.

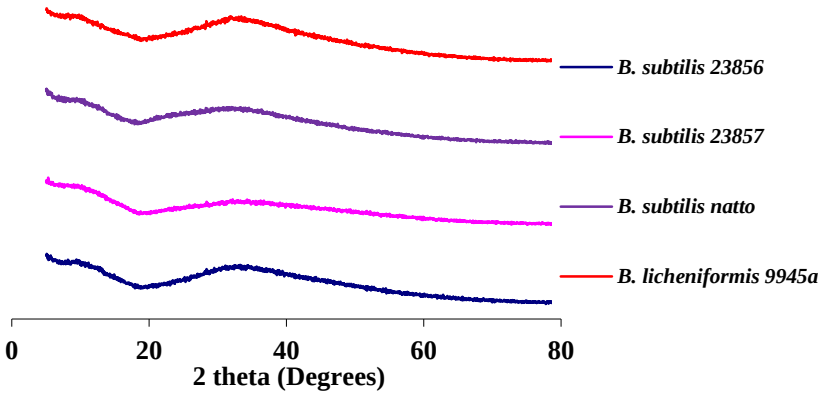


Fig 4.13: Crystallinity of γ-PGA produced by different *Bacillus* strains in Medium E. Crystallinity was assessed by performing XRD spectroscopy (Phillips PW1700). Experiments were conducted in triplicate. Each XRD spectrum is a mean of 3 spectra.

4.7 Molecular weight

As mentioned previously (see **section 1.6**), γ-PGA can be produced with different molecular weights. The molecular weight is an important characteristic of any polymer produced, since it can directly affect the application for which it is used. For instance, lower molecular weight γ-PGA can be used for its antifreeze activity (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003), whereas a higher molecular weight polymer is required for waste water treatment applications (Inbaraj *et al.*, 2006; Sung *et al.*, 2005). Analysing the molecular weight of γ-PGA would also demonstrate whether it is dependent on bacteria or medium of production or both. Another important characteristic of the polymer is its polydispersity (Pd). The closer the value of Pd to unity, the more homogenous the polymer is with respect to its molecular weight.

The molecular weight and Pd for γ-PGA produced by eight bacteria in GS and E media was determined using aqueous based GPC. When grown in GS medium, *B. licheniformis* 1525, *B.*

licheniformis 6816 and *B. subtilis* natto produced high molecular weight γ-PGA (**Table 4.2**). The other strains under study produced a lower molecular weight product (~3000 Da).

Table 4.2: Molecular weight of γ-PGA produced by bacteria grown in GS medium at 37˚C & 150 rpm for 96 h. Molecular weight was analysed using GPC (MZ Hema guard plus 2 x Hema Linear column). Experiments were conducted in triplicate (n = 3).

Strain name	Molecular weight (Da) (M_w)	Molecular number (M_n)	Polydispersity (M_w/M_n)
B. licheniformis 1525	871000	647000	1.35
B. licheniformis 6816	850000	596500	1.45
***B. subtilis* natto**	257000	54550	4.75

In medium E, *B. licheniformis* 6816 and *B. subtilis* natto produced γ-PGA with a high molecular weight (**Table 4.3**). *B. licheniformis* 1525, which produced a high molecular weight polymer in GS medium, produced a low molecular weight polymer in medium E. The remaining bacterial strains under study also produced a low molecular weight product (< 3000 Da).

Table 4.3: Molecular weight of γ-PGA produced by bacteria in Medium E. Molecular weight analysis was performed using GPC (MZ Hema guard plus 2 x Hema Linear column). Experiments were conducted in triplicate (n = 3).

Strain name	Molecular weight (Da) (M_w)	Molecular number (M_n)	Polydispersity (M_w/M_n)
***B. licheniformis* 1525**	< 3000	-	-
B. licheniformis 6816	856500	715500	1.2
B. subtilis natto	760000	605000	1.3

4.8 Summary of γ-PGA production in shake flasks

When γ-PGA production in eight *Bacillus* strains grown in GS and E media was investigated, it was observed that all bacteria produced a white polymer when grown in GS medium and a brown polymer when grown in medium E. The produced polymer was identified as γ-PGA using FT-IR analysis, where the spectra for γ-PGA produced by the eight *Bacillus* strains showed all the characteristic peaks and compared well to the FT-IR spectrum of a commercially available γ-PGA.

Samples were taken at regular intervals during shake flask culture for growth and nutrient consumption analysis. **Fig 4.14** shows the growth of all bacterial strains in GS and E medium. At 0 h, all bacteria had a cell count of ~5 log CFU/ml. Amongst all bacteria under study, in GS medium, *B. subtilis* 23858 reached the highest cell count of 9.58 log CFU/ml at 72 h (**Fig 4.14a**). In medium E, *B. licheniformis* 1525 reached a maximum cell count of 8.57 log CFU/ml at 72 h (**Fig 4.14b**). In medium E, the maximum cell counts for the five *B. subtilis* strains (~7.5-8.1 log CFU/ml) were not as high as those observed with the *B. licheniformis* strains (~8.5 log CFU/ml). *B. subtilis* 23858 & 23859 and *B. licheniformis* 1525, 6816 & 9945a had a higher cell count at 96 h in GS medium than in medium E. In fact, *B. subtilis* 23859 did not grow well in medium E, with a maximum cell count of 7.55 log CFU/ml after 24 h and a cell count of ~ 4.9 log CFU/ml at the end of 96 h. It was also evident that each individual bacterial strain reached a higher cell count in GS medium than in medium E using shake flasks.

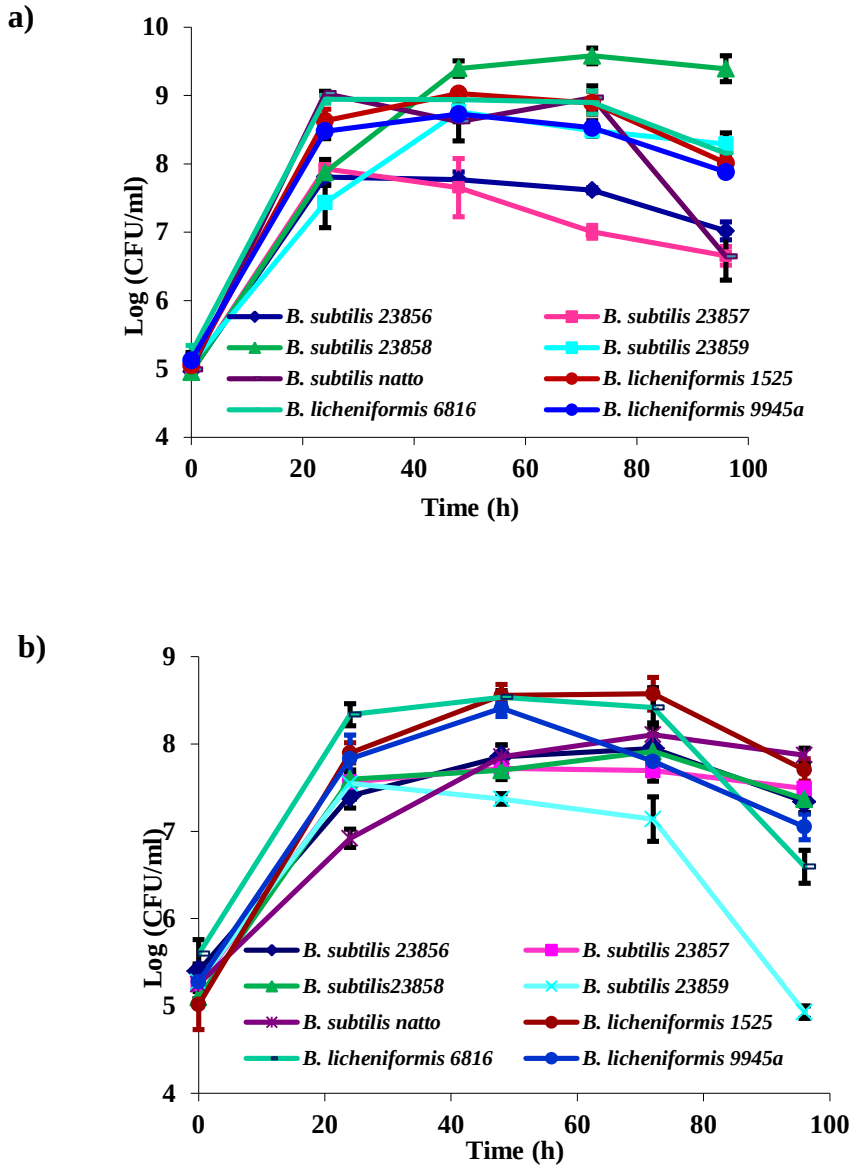


Fig 4.14: Growth curves for 8 *Bacillus* strains when grown in a) GS medium & b) medium E at 37˚C & 150 rpm for 96 h. Experiments were conducted in triplicate (n = 3).

Table 4.4 shows the decrease in sucrose and L-glutamic acid concentration in GS medium at the end of the shake flask culture of the eight bacteria, along with the maximum cell count observed and yield of γ-PGA produced after 96 h. In GS medium, all strains utilized more than 84% of the sucrose provided. The least amount of sucrose was consumed by *B. subtilis* 23859 (84.32%, $P < 0.05$), whereas *B. licheniformis* 6816 utilized sucrose the most (97.96%,

P < 0.05). In GS medium, *B. subtilis* strains consumed more L-glutamic acid than *B. licheniformis* strains. *B. subtilis* natto consumed the most L-glutamic acid at the end of 96 h (~95%, P < 0.05) whereas *B. licheniformis* 9945a utilized only 17.46% of the provided L-glutamic acid. Incidentally, *B. subtilis* natto also produced the highest yield of γ-PGA in GS medium (~17.7g/l, P < 0.05).

Table 4.4: Nutrient utilization, maximum viable cell count and yield of γ-PGA for 8 bacteria when grown in GS medium at 37°C/150 rpm for 96 h. Experiments were conducted in triplicate (n = 3).

Parameters → / Bacteria ↓	Sucrose (g/l)	L-glutamic acid (g/l)	Maximum cell count (Log CFU/ml)	Yield (g/l)	Yield (ng/cell)
***B. subtilis* 23856**	45.33 ± 0.88	12.94 ± 0.42	7.80 ± 0.10	13.55 ± 0.45	0.21
***B. subtilis* 23857**	44.09 ± 0.96	11.70 ± 0.26	7.93 ± 0.07	16.51 ± 0.20	0.19
***B. subtilis* 23858**	45.38 ± 0.71	16.45 ± 0.00	9.58 ± 0.11	14.88 ± 0.48	0.004
***B. subtilis* 23859**	42.16 ± 0.55	13.45 ± 0.91	8.76 ± 0.05	16.03 ± 0.03	0.03
***B. subtilis* natto**	44.97 ± 2.05	19.00 ± 0.09	9.03 ± 0.03	17.77 ± 0.52	0.02
***B. licheniformis* 1525**	47.38 ± 0.61	7.84 ± 2.43	9.02 ± 0.02	15.93 ± 0.18	0.02
***B. licheniformis* 6816**	48.98 ± 1.80	6.77 ± 3.40	8.95 ± 0.06	13.3 ± 0.30	0.01
***B. licheniformis* 9945a**	43.49 ± 1.83	3.49 ± 0.81	8.73 ± 0.11	14.05 ± 0.18	0.03

The three *B. licheniformis* strains utilized more glycerol in medium E, than the *B. subtilis* strains under study (**Table 4.5**). Glycerol in medium E was consumed better than sucrose was in GS medium by the three *B. licheniformis* strains and *B. subtilis* 23859 (P < 0.05). The remaining strains preferred sucrose as the C source. *B. licheniformis* 1525 reached the highest maximum cells count (8.57 log CFU/ml) and also produced the highest yield of γ-PGA (22.3 g/l) in medium E (P < 0.05). *B. subtilis* natto utilized 14.34 g/l more of L-glutamic acid in GS medium than that in medium E. It also produced the lowest yield of γ-PGA in medium E (5.7 g/l, P < 0.05). Interestingly, all bacteria produced more γ-PGA/cell in medium E than in GS medium.

Table 4.5: Nutrient utilization, maximum viable cell count and yield of γ-PGA for 8 bacteria when grown in medium E at 37°C/150 rpm for 96 h. Experiments were conducted in triplicate (n = 3).

Parameters → / Bacteria ↓	Glycerol (g/l)	L-glutamic acid (g/l)	Maximum cell count (Log CFU/ml)	Yield (g/l)	Yield (ng/cell)
***B. subtilis* 23856**	39.91 ± 1.23	4.89 ± 0.07	7.41 ± 0.14	12.93 ± 1.11	0.5
***B. subtilis* 23857**	43.34 ± 0.46	5.94 ± 0.26	7.72 ± 0.05	21.75 ± 0.05	0.41
***B. subtilis* 23858**	40.20 ± 0.92	5.70 ± 0.23	7.91 ± 0.06	19.32 ± 0.37	0.24
***B. subtilis* 23859**	50.38 ± 0.59	5.26 ± 0.23	7.55 ± 0.08	21.15 ± 0.07	0.60
***B. subtilis* natto**	39.65 ± 0.85	4.66 ± 0.05	8.11 ± 0.14	5.7 ± 0.35	0.04
***B. licheniformis* 1525**	52.52 ± 1.27	6.84 ± 0.27	8.57 ± 0.19	22.30 ± 0.12	0.06
***B. licheniformis* 6816**	54.07 ± 0.77	8.89 ± 0.41	8.54 ± 0.07	12.70 ± 0.45	0.04
***B. licheniformis* 9945a**	56.53 ± 0.73	7.20 ± 0.16	8.41 ± 0.09	12.58 ± 0.24	0.05

A summary of the properties of γ-PGA obtained with the 8 *Bacillus* strains grown in GS and E medium is presented in **Tables 4.6 & 4.7**. It was seen that all bacteria produced a crystalline polymer when grown in GS medium. In contrast, amorphous γ-PGA was obtained when bacteria were grown in medium E. Most of the γ-PGA produced by bacteria in GS medium was the sodium salt of the polymer (Na-γ-PGA), whereas the acid form of γ-PGA (H^+-γ-PGA) was also produced when bacteria were grown in medium E. Hence, crystallinity and form of γ-PGA were dependent on medium of γ-PGA production. On the other hand, the molecular weight of γ-PGA produced was seen to be dependent on the medium and the bacterial strain producing it. Although *B. licheniformis* 6816 produced γ-PGA with similar molecular weight in GS and E media, the molecular weights of γ-PGA produced by *B. subtilis* natto and *B. licheniformis* 1525 in GS and E medium were different.

This study has contributed 6 bacteria that have not been previously used for γ-PGA production. In addition, the γ-PGA produced by all of the 8 bacteria investigated was characterized in terms of its yield, form, crystallinity and molecular weight.

Table 4.6: Summary of properties of γ-PGA produced from 8 *Bacillus* strains in GS medium.

Organism	Yield (g/l)	Crystallinity	% Na-γ-PGA	Molecular weight (Da)	No. of glutamic acid monomers
B. subtilis 23856	13.55 ± 0.78	Crystalline	86.90	~ 3000	~20
B. subtilis 23857	16.52 ± 0.35	Crystalline	87.55	~ 3000	~20
B. subtilis 23858	14.88 ± 0.84	Crystalline	80.26	~ 3000	~20
B. subtilis 23859	16.03 ± 0.06	Crystalline	82.40	~ 3000	~20
***B. subtilis* natto**	17.77 ± 0.9	Crystalline	85.38	257500 (Pd = 4.75)	~1752
B. licheniformis 1525	15.93 ± 1.16	Crystalline	97.40	871000 (Pd = 1.35)	~5925
B. licheniformis 6816	13.3 ± 0.52	Crystalline	88.37	850000 (Pd = 1.45)	~5782
***B. licheniformis* 9945a**	14.05 ± 0.31	Crystalline	90.30	~ 3000	~20

Table 4.7: Summary of properties of γ-PGA produced from 8 *Bacillus* strains in medium E.

Organism	Yield (g/l)	Crystallinity	% Na-γ-PGA	% H^+-γ-PGA	Molecular weight (Da)	No. of glutamic acid monomers
B. subtilis 23856	12.93 ± 0.07	Amorphous	59.90	37.76	<3000	~20
B. subtilis 23857	21.75 ± 0.34	Amorphous	52.68	44.98	<3000	~20
B. subtilis 23858	19.32 ± 0.09	Amorphous	56	41.91	<3000	~20
B. subtilis 23859	21.15 ± 0.12	Amorphous	56.29	41.73	<3000	~20
***B. subtilis* natto**	5.7 ± 0.25	Amorphous	52.31	44.56	760000 (Pd = 1.3)	5170
B. licheniformis 1525	22.3 ± 0.15	Amorphous	50.32	48.09	<3000	~20
B. licheniformis 6816	12.7 ± 0.45	Amorphous	38.84	57.97	856500 (Pd = 1.2)	~5827
***B. licheniformis* 9945a**	12.58 ± 0.24	Amorphous	64.47	32.89	<3000	~20

After identification and characterization of the polymer produced by eight bacteria in GS and E media (**see Tables 4.6 & 4.7**), it was evident that *B. subtilis* natto would be a good choice for the probiotic food application. This is because *B. subtilis* natto is food derived (natto is a traditional Japanese food item) and hence, it can be used for a food application. Although, all the eight bacteria under study produced a sodium salt of γ-PGA in GS medium, which is

desirable for the probiotic research undertaken in this study, because it is known to have good antifreeze properties (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003), *B. subtilis* natto produced the highest yield of γ-PGA in GS medium compared to the other bacteria and hence, it would provide the highest quantities of Na-γ-PGA for probiotic tests. Also, γ-PGA produced by *B. subtilis* natto in GS medium was white in colour, which is a desirable property for additives to food products.

It was essential to scale up the production of γ-PGA by *B. subtilis* natto from shake flasks to a fermenter, because a higher yield of γ-PGA could be produced when bacteria are grown in a fermenter, where the parameters that affect cell growth and γ-PGA production can be controlled and monitored. In addition, by increasing the volume of production, the total quantity of γ-PGA that would be produced in a fermenter would also be higher than that in shake flasks. The next section will describe the production of γ-PGA using *B. subtilis* natto grown in GS medium in 4 l fermentations.

5. RESULTS – BACTERIAL γ-PGA PRODUCTION - FERMENTATION CULTURE

5.1 Introduction

On investigating the properties of γ-PGA produced by eight bacteria in two different media, γ-PGA produced by *B. subtilis* natto grown in GS medium was chosen for testing a novel probiotic application (See **Section 4.8**). For testing the effect of γ-PGA on the viability of probiotic bacteria, scaling up γ-PGA production was important to increase the yield of γ-PGA produced by *B. subtilis* natto (when grown in a more controlled environment than shake flasks) and for increasing the total quantity of γ-PGA produced so that enough γ-PGA was available for testing the probiotic application. For this purpose, cellular growth and γ-PGA production by *B. subtilis* natto was studied during growth in 4 l fermentations (Electrolab batch fermenter) where temperature, pH, agitation, aeration and dissolved O_2 could be controlled.

5.2 Fermentation of *B. subtilis* natto to produce γ-PGA

Fermentations were run for 96 h and parameters that can affect the growth of cells and production of γ-PGA were monitored and controlled. It was seen that dO_2 decreased to 40% saturation within the first 10 h of growth, after which it was maintained at that value by controlling agitation and aeration rates. The control of dO_2 by maintaining agitation rates can be seen in **Fig 5.1a**. The pH of the culture was maintained at 6.8 throughout the fermentation by addition of 3 M HCl and 3 M NaOH as required (**Fig 5.1b**). Temperature was controlled at 37˚C throughout the 96 h fermentation run.

When grown in 4 l fermentations, *B. subtilis* natto reached a maximal cell count of 10.11 log CFU/ml at 72 h (**Fig 5.1c**), which subsequently decreased to 9.23 log CFU/ml over 96 h. The yield of γ-PGA produced by *B. subtilis* natto by the end of fermentation was 27.94 g/l ± 0.81 g/l.

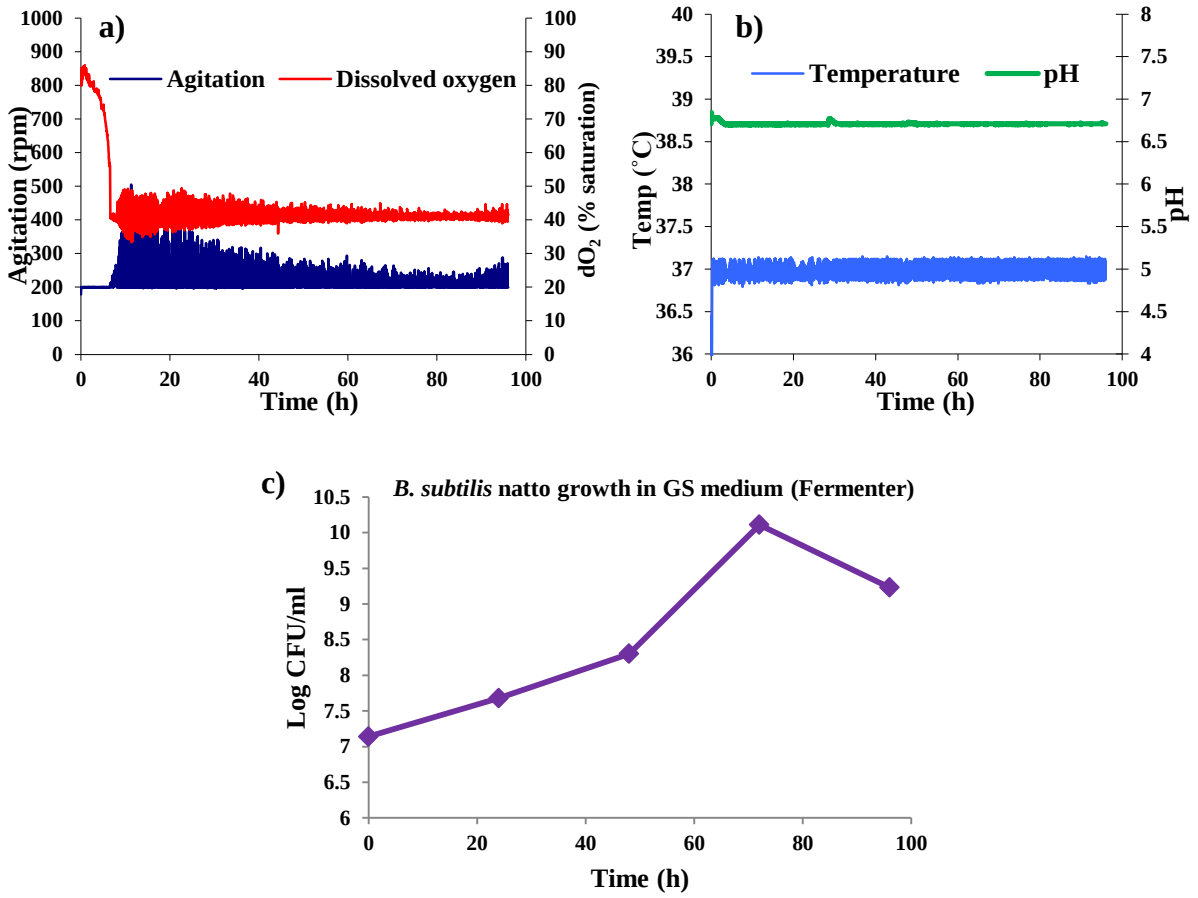


Fig 5.1: a) Agitation & dO_2, b) temperature & pH and c) growth during fermentation of *B. subtilis* natto to produce γ-PGA in a 5 l fermenter. Cells were grown at 37°C for 96 h in GS medium, after which γ-PGA was recovered from the medium.

5.3 Analysis of γ-PGA obtained from *B. subtilis* natto grown in a fermenter

γ-PGA obtained from growth of *B. subtilis* natto in a 4 l fermentation was purified using dialysis and both crude and purified γ-PGA were identified using FT-IR spectroscopy. This analysis was performed to confirm that the produced polymer was γ-PGA and to identify differences (if any) in the spectra of crude and pure γ-PGA. The FT-IR spectra of the γ-PGA produced by fermentation were plotted as before. Crude γ-PGA showed peaks at 3292 cm^{-1}, 1564 cm^{-1}, 1401 cm^{-1}, 1133 cm^{-1}, 854 cm^{-1} and 616 cm^{-1} which corresponded to carboxyl, amide II stretch, C=O symmetric stretch, C-N stretch, C-H stretch and N-H oop bending respectively (**Fig 5.4**). Purified γ-PGA showed peaks at 3263 cm^{-1}, 1535 cm^{-1}, 1393 cm^{-1},

1165 cm^{-1}, 891 cm^{-1} and 668 cm^{-1} which represent the presence of hydroxyl, amide II stretch, C=O symmetric stretch, C-N stretch, C-H stretch and N-H out-of-plane bending respectively. In addition, purified γ-PGA also showed peaks at 1641 cm^{-1} and 1236 cm^{-1}, which correspond to amide I N-H bending and C-O stretch respectively. The additional peaks that are present on the spectra of γ-PGA purified using dialysis could be used to identify the polymer better.

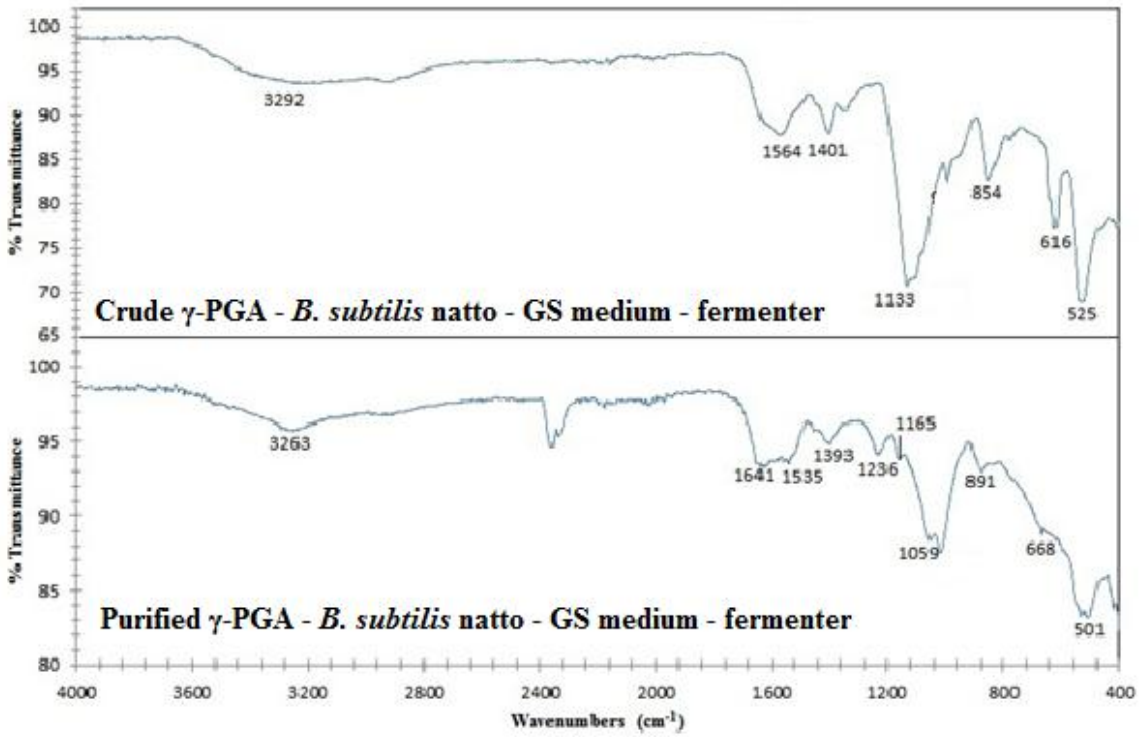


Fig 5.4: FT-IR spectra for crude and purified (dialyzed) γ-PGA produced by fermentation of *B. subtilis* natto. Each FT-IR spectrum is a mean of 3 spectra.

In addition, the crude and purified γ-PGA were analysed using ICP-AES, to determine the difference in salt content of γ-PGA. It was seen that crude γ-PGA had a high concentration of Na ions (910.27 ppm). However, when purified, the same sample of γ-PGA showed the presence of a lower concentration of sodium ions (340.10 ppm). Similarly, crude γ-PGA had 27.33 ppm of K, whereas pure γ-PGA only had 15.06 ppm of K. The lower concentration of these ions in purified γ-PGA indicates that purification using dialysis eliminated lower

molecular weight impurities and hence purification is an important step before γ-PGA is used for subsequent applications.

6. RESULTS – PROTECTIVE EFFECT OF γ-PGA ON PROBIOTIC BACTERIA

6.1 Introduction

γ-PGA was produced using *B. subtilis* natto grown in GS medium in 4 l fermentations. The produced polymer was isolated from growth media, purified, identified and characterized. This polymer was then used for novel probiotic applications to protect cells during different conditions. Firstly, the protective effect of this polymer on the viability during freeze drying of three probiotic bacteria was tested. Secondly, there is a need to come up with probiotic delivery platforms other than dairy products. Fruit juices are a potential alternative, but introduction of probiotic bacteria into fruit juices is challenging, since they present more stressful conditions to the bacteria, resulting in a loss in viability. Therefore, two *Bifidobacteria* strains were protected with γ-PGA and introduced in orange and pomegranate juice to test their survival. Finally, there is heavy loss in cell viability when probiotic bacteria pass through the harsh conditions of the stomach. Hence, γ-PGA was used to protect two *Bifidobacteria* strains in simulated gastric juice for 4 h.

6.2 Sterilization of γ-PGA

Sterilization of γ-PGA is important because there is a need to eradicate any residual bacteria from the fermentation that might interfere with subsequent viable cell count results for the probiotic bacteria. Therefore, different sterilization techniques were tested to identify the most appropriate method to effect sterilization, but without substantially disrupting the structure of γ-PGA. The results of the techniques used are shown in **Table 6.1**.

Autoclaving γ-PGA at 0.35 BAR at 110˚C for 30 mins was obviously the more preferred method of sterilization since it exposed the polymer to less stressful conditions than autoclaving γ-PGA at 1.035 BAR at 121˚C for 20 mins.

Table 6.1: Various methods used to sterilize γ-PGA and their outcomes.

Sterilization method	Outcome	Comments
Filtration through 0.45 µ filter	Not effective	10% γ-PGA solution too viscous to pass through filter
Ethanol treatment	Not effective	Does not kill spores of *B. subtilis*
U.V.	Not effective	Only surface sterilization achieved.
3% Hydrogen peroxide	Effective	Effectively sterilizes γ-PGA, but could have adverse effects on γ-PGA structure. Cannot completely get rid of peroxide.
Autoclaving at 1.035 BAR at 121˚C for 20 mins	Effective	Could have adverse effects on structure and functionality of γ-PGA. Need to check with FT-IR.
Autoclaving at 0.35 BAR at 110˚C for 30 mins	Effective	Could have adverse effects on structure and functionality of γ-PGA. Need to check with FT-IR.

The effect of autoclaving (0.35 BAR at 110˚C for 30 mins) on the characteristic bonds of γ-PGA was tested using FT-IR. γ-PGA samples sterilized by autoclaving and with hydrogen peroxide treatment were compared to a non-sterile γ-PGA sample using FT-IR to determine any disruption in functional groups because of the sterilization technique (**Fig 6.1**).

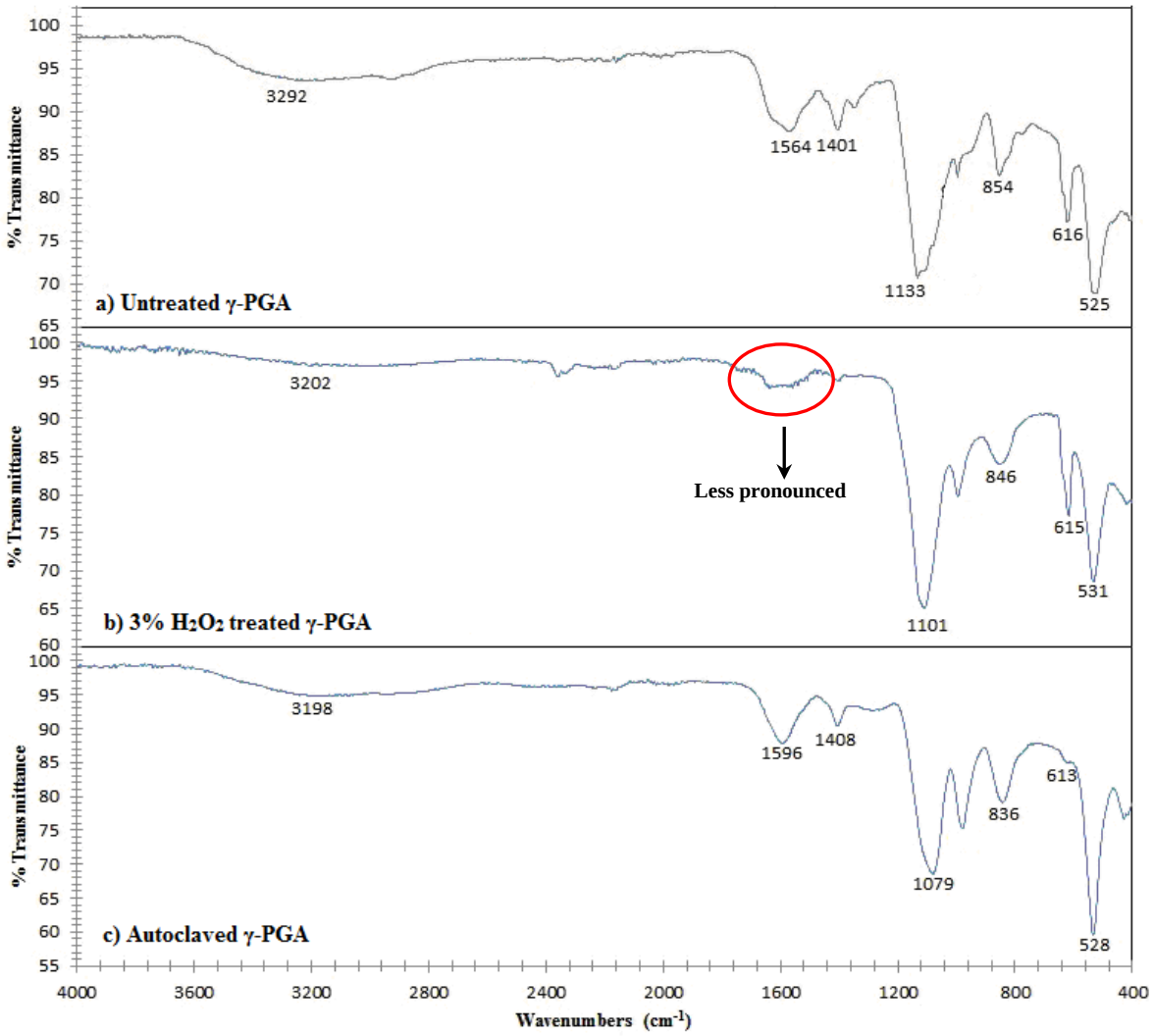


Fig 6.1: FT-IR spectra of a) untreated γ-PGA b) 3% H_2O_2 treated γ-PGA and c) autoclaved γ-PGA (0.35 BAR at 110˚C for 30 mins) produced by *B. subtilis* natto grown in GS medium in a 5 l fermenter. Each FT-IR spectrum is a mean of 3 spectra.

It was seen that the spectrum for autoclaved γ-PGA compared well to that of the untreated γ-PGA and showed the presence of peaks corresponding to all the previously mentioned characteristic functional groups (see **Fig 6.1**). However, whilst the spectrum for γ-PGA treated with 3% H_2O_2 showed all the peaks present in the non-sterile sample, it was noted that peaks at 1564 cm^{-1} and 1401 cm^{-1} (corresponding to amide II stretch and C=O symmetric stretch respectively) were less pronounced in γ-PGA samples treated with peroxide (see

circled region in **Fig 6.1**). This could mean that peroxide treatment might have caused some disruption within these functional groups.

Because of the results obtained above, autoclaving γ-PGA at 0.35 BAR at 110˚C for 30 min was the chosen method for sterilizing γ-PGA. γ-PGA sterilized using this method will henceforth be referred to as sterilized γ-PGA or γ-PGA[S]. Likewise, γ-PGA that has not been sterilized will be referred to as untreated γ-PGA or γ-PGA[U].

6.3 Use of γ-PGA as cryoprotectant for 3 probiotic bacteria

After assessing different sterilization techniques, autoclaving at 110˚C at 0.35 BAR for 30 mins was chosen to sterilize γ-PGA for probiotic tests. Sterilized γ-PGA (γ-PGA[S]) and untreated γ-PGA (γ-PGA[U]) were used to coat probiotic bacteria and the cryoprotectant properties were evaluated by comparison to sucrose as a cryoprotectant during freeze drying. This is a novel application, since γ-PGA has never been used to protect probiotic bacteria during freeze drying.

6.3.1 Testing γ-PGA[S] as cryoprotectant for 3 probiotic bacteria

γ-PGA[S] was first tested as a cryoprotectant for probiotic bacteria. Three commonly used probiotic bacteria (*L. paracasei*, *B. breve* and *B. longum*) were used for the tests. 10% sucrose has been shown to offer better protection during freeze drying of probiotic *Lactobacilli* when compared to trehalose and sorbitol (Siaterlis *et al.*, 2009). Hence, the effect of 10% γ-PGA[S], 5% γ-PGA[S] and 10% sucrose was tested on viability of the bacteria before and after freeze drying (**Fig 6.2**). On conclusion of these tests, it was observed that when no cryoprotectant was used to protect the cells, *L. paracasei* showed a reduction in viability of 1.34 log CFU/ml. When 10% sucrose was used to protect *L. paracasei* during freeze drying, 0.91 log CFU/ml reduction in viability was observed. However, for 10% γ-PGA[S]-protected cells, loss in viability was reduced to 0.51 log CFU/ml. For *L. paracasei*, 10% γ-PGA[S] was able to protect the cells significantly better than 10% sucrose ($P \leq 0.05$). The cryoprotectant ability of 5% γ-PGA[S] was comparable to, but not significantly different ($P > 0.05$) from 10% sucrose.

A more pronounced reduction in viability (2.47-2.52 log CFU/ml) was observed when both *Bifidobacteria* strains were freeze dried without any cryoprotectant. When the cells were

protected with 10% γ-PGA[S], only 1.24-1.26 log CFU/ml reduction in viability was observed. The cryoprotectant ability of 10% sucrose and 10% γ-PGA[S] for *Bifidobacteria* was comparable ($P \geq 0.05$).

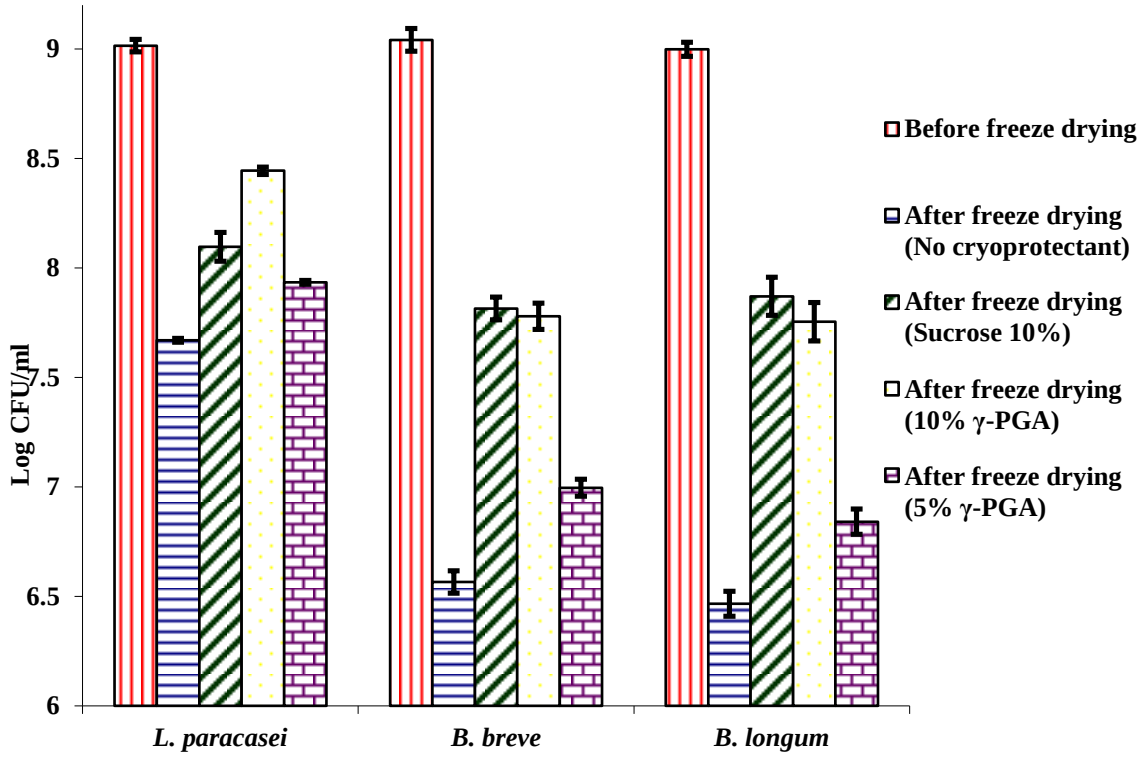


Fig 6.2: Effect of γ-PGA[S] and sucrose on viability of probiotic bacteria during freeze drying. Cells were freeze dried at -40˚C and 5 MBAR pressure and viability was measured before and after freeze drying on TPY agar. Experiments were conducted in triplicate (n = 3).

6.3.2 Testing γ-PGA[U] as a cryoprotectant for 3 probiotic bacteria

On conclusion of tests with γ-PGA[S] as a cryoprotectant, it was important to ascertain whether autoclaving affected the cryoprotectant abilities of γ-PGA. Even though it was confirmed that autoclaving did not affect the structure of γ-PGA, it could have affected its molecular weight. Molecular weight, in turn, can have an effect on γ-PGA as a cryoprotectant. Hence, γ-PGA[U] was also tested for its ability to protect cells during freeze drying. From the previous tests, it was evident that *Bifidobacteria* were more sensitive than *Lactobacillus*. Hence, only the former were used for the tests.

The viable plate counts for tests with γ-PGA[S] were done on TPY agar, which is a non-selective media for enumeration of *Bifidobacteria*. This could be done because pressure cooking eliminated any residual bacteria in γ-PGA. For tests with γ-PGA[U], it was important to use a selective and differential media for enumeration, which would inhibit the growth of other bacteria while differentiating *Bifidobacteria*. Hence, BSM agar was used. *Bifidobacteria* grown on BSM agar plates were a purplish-brown colour. When a 10% solution of γ-PGA[U] was plated onto BSM agar and incubated for 24-48 h under anaerobic conditions, no colonies were observed. To compare the results of cryoprotectant tests with γ-PGA[S] & γ-PGA[U], it was important to compare the counts of the same *Bifidobacteria* culture on TPY and BSM and check if they were comparable (**see Table 6.2**). It was seen that 22 h and 16 h cultures of *B. breve* and *B. longum* showed similar counts on TPY and BSM, hence the viable cell count was not significantly different ($P > 0.05$). Thus, it was concluded that BSM could be used for tests with γ-PGA[U].

Table 6.2: Comparison of growth of *Bifidobacteria* on TPY and BSM when incubated anaerobically. *B. breve* were incubated for 22 h and *B. longum* were incubated for 16 h.

Organism	Count on TPY (Log CFU/ml)	Count on BSM (Log CFU/ml)
Bifidobacterium breve	**8.55±0.15**	**8.48±0**
Bifidobacterium longum	**8.52±0.18**	**8.60±0.12**

The effect of 10% γ-PGA[U] was then tested on the viability of *Bifidobacteria* during freeze drying and was compared with the cryoprotective ability of 10% γ-PGA[S] (**Fig 6.3**). Similar to the tests with γ-PGA[S], a log reduction of ~2.82 CFU/ml was observed when cells were freeze dried without any cryoprotectant. When cells were protected using 10% γ-PGA[U], a log reduction of 1.92-1.99 CFU/ml was observed. However, when 10% γ-PGA[S] was used to protect *Bifidobacteria* during freeze drying, 1.34-1.38 log CFU/ml reduction in viability was observed. Hence, although 10% γ-PGA[U] could protect the cells during freeze drying as

compared to when no cryoprotectant was used, it was not as effective ($P < 0.05$) as 10% γ-PGA[S]. Thus, sterilization using autoclaving had the added advantage of improving the cryoprotectant ability of γ-PGA.

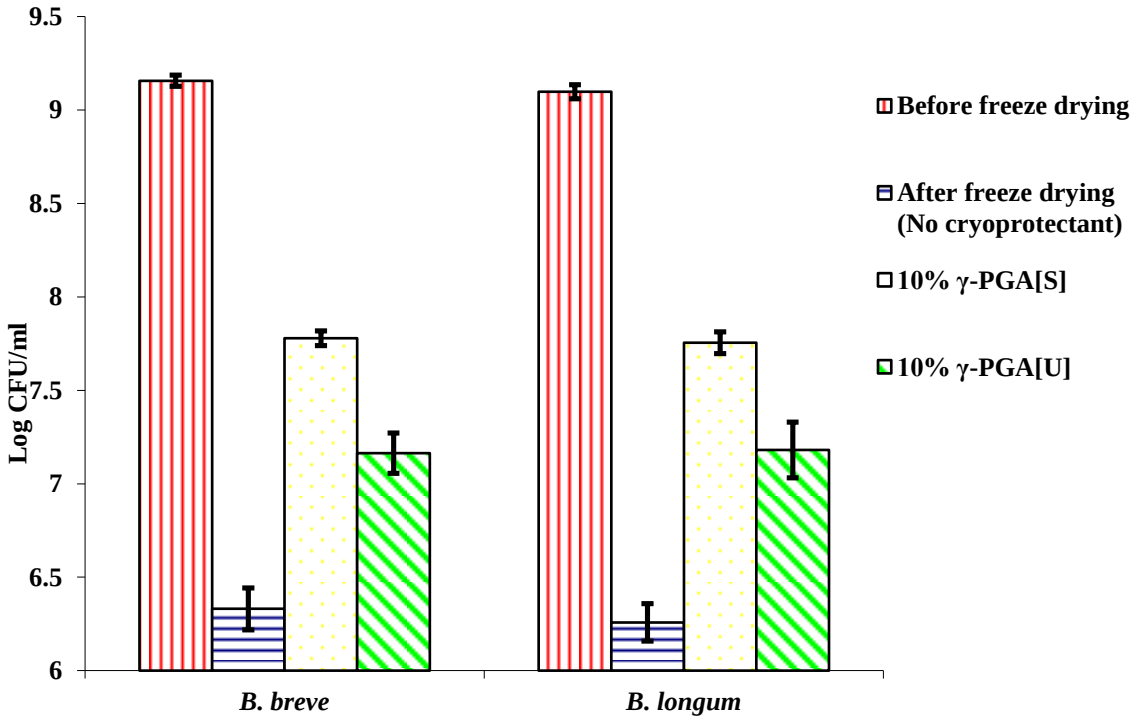


Fig 6.3: Effect of γ-PGA[S] & γ-PGA[U] on viability of probiotic bacteria during freeze drying. Cells were freeze dried at -40˚C and 5 MBAR pressure and viability was measured before and after freeze drying on BSM agar. Experiments were conducted in triplicate (n = 3).

6.3.3 SEM analysis

Freeze dried powders containing unprotected *Bifidobacteria* and *Bifidobacteria* protected with γ-PGA[S] were analysed using SEM to understand how the cells may be protected by embedding within the polymer. It should be noted that SEM analysis only shows a surface image. As is evident from **Figs 6.4a & 6.4b**, freeze dried *B. longum* cells protected with γ-PGA appear to be encapsulated within a thin layer of γ-PGA. The thickness of γ-PGA coating could be calculated with further testing using transmission electron microscopy (TEM). In addition, cells are also seen embedded within the γ-PGA matrix.

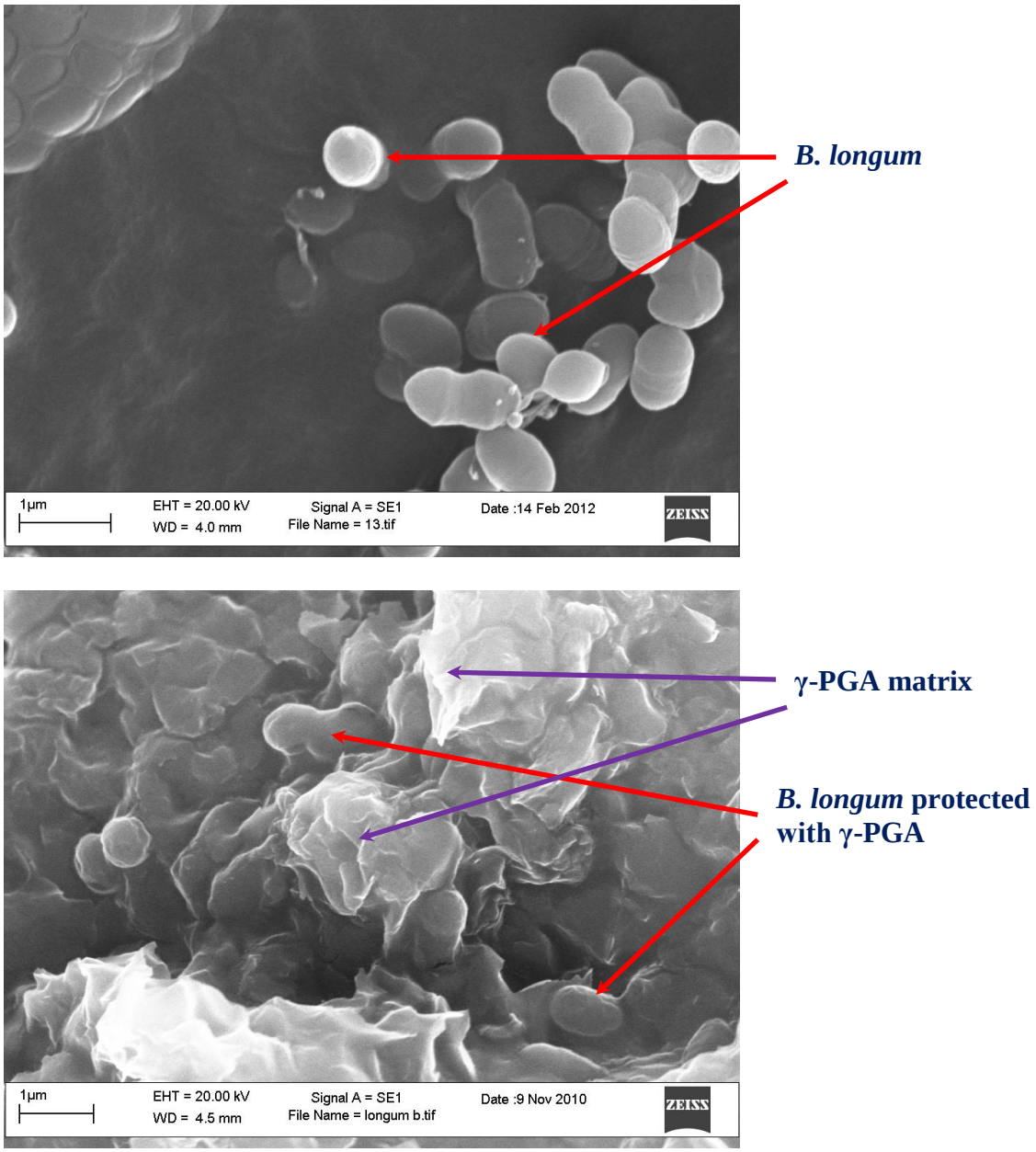


Fig 6.4: SEM image of a) Freeze dried *B. longum* cells b) Freeze dried *B. longum* protected with γ-PGA. SEM analysis was performed using Zeiss EVO50, U.K. and photographs were analysed using the software provided by Zeiss EVO50.

6.4 γ-PGA protection of *Bifidobacteria* in orange and pomegranate juice

After confirming that γ-PGA can be used as a cryoprotectant for probiotic bacteria to reduce their loss in viability during freeze drying, γ-PGA was tested for its effect on the viability of two probiotic bacteria during storage in orange and pomegranate juice. This work is important, because there is a need to develop non-dairy based foods that can deliver probiotic bacteria. Juices are a good platform for this purpose. However, the survival conditions that the bacteria are exposed to in fruit juices are less favourable than those in dairy products. Therefore, it is important to come up with a technology that can help probiotic bacteria survive in these juices during their shelf life. γ-PGA has never been used for protection of probiotic bacteria in fruit juices and hence, this is a novel application.

6.4.1 Use of BSM for enumeration of bacteria in orange and pomegranate juice

Before testing γ-PGA with probiotic bacteria, it was important to identify the best medium that could be used for determining the viability of the bacteria. Commercial orange and pomegranate juices are pasteurized during manufacture. This means that although the juice is free of spoilage/disease causing bacteria, it may not be completely free of other residual bacteria. Therefore, for probiotic juice tests, it was essential to use a selective and differential media for enumeration of *Bifidobacteria*, hence BSM agar was used for this purpose. When Tropicana orange juice was plated onto TSA and incubated at 37˚C for 24 h, residual bacteria in the juice appeared on the plate in the form of colonies. However, when the same juice was plated on BSM, no growth was seen, thus confirming that the residual bacteria in orange juice would not interfere with *Bifidobacteria* counts, when they are introduced into the juice. Similar results were obtained with pomegranate juice that was used for the study.

6.4.2 Protection of probiotic bacteria in orange juice

Once BSM was identified as the appropriate medium for enumeration of probiotic bacteria for these tests, γ-PGA-protected *B. longum* and *B. breve* were introduced into orange juice and stored at 4˚C. Viability was measured at regular intervals.

6.4.2.1 *B. longum*

The effect of 2.5% γ-PGA on the viability of *B. longum* in orange juice for 39 days was tested (**Fig 6.5**). Bacteria that were not protected with γ-PGA failed to survive in orange juice for 20 days. In contrast, γ-PGA-protected cells survived well for 39 days with a viability of 6.48 log CFU/ml, when compared to their initial count of 9.47 log CFU/ml.

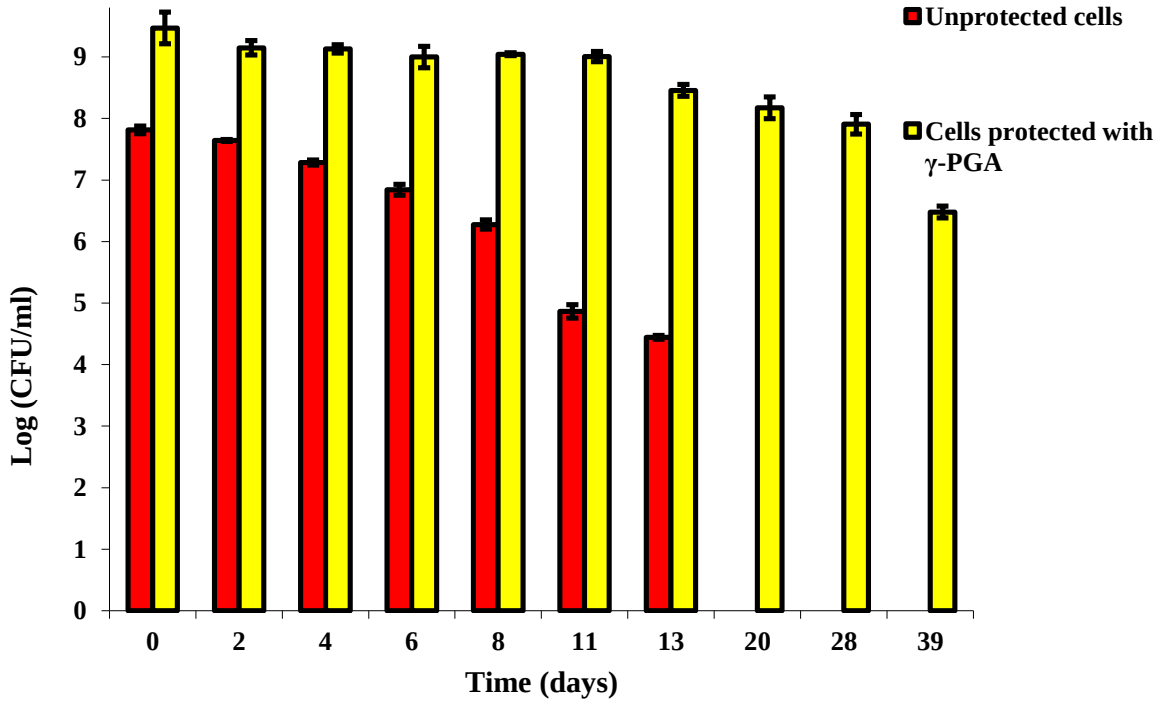


Fig 6.5: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. longum* in orange juice. Unprotected and γ-PGA protected cells were introduced into fresh orange juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

A log reduction of 3.37 CFU/ml was seen in viability of *B. longum* cells that were not protected with γ-PGA after 13 days, whereas a log reduction of only 1.10 CFU/ml was observed for γ-PGA-protected cells. On day 20, unprotected *B. longum* showed complete loss in viability. Even on day 11, the number of unprotected cells was much less (4.86 log CFU/ml) than the recommended FAO value of 6-7 log CFU/ml (Kurmann and Rasic, 1991).

6.4.2.2 *B. breve*

The effect of 2.5% γ-PGA was also tested on viability of *B. breve* when stored in orange juice for 39 days (**Fig 6.6**). Similar to tests with *B. longum*, cells that were not protected with γ-PGA failed to survive in orange juice for 20 days. In contrast, γ-PGA protected cells survived well for 39 days with a viability of 6.61 log CFU/ml, when compared to their initial count of 8.66 log CFU/ml. γ-PGA protected *B. breve* cells showed only 0.54 log reduction in viability after 13 days in comparison to a 3.97 log decrease in viability for unprotected cells. On day 20, unprotected *B. breve* cells showed complete loss in viability. Even on day 11, the number of unprotected cells was much less (4.5 log CFU/ml) than the recommended value of 6-7 log CFU/ml by the FAO (Kurmann and Rasic, 1991).

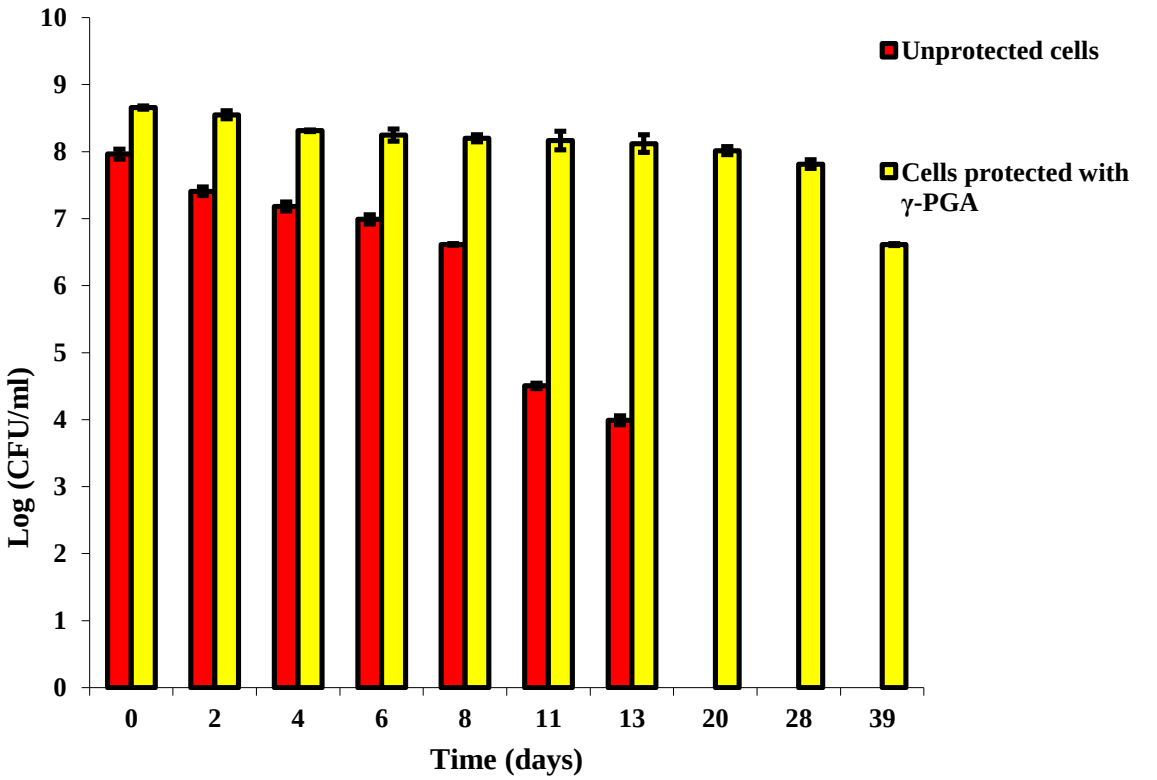


Fig 6.6: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. breve* in orange juice. Unprotected and γ-PGA protected cells were introduced into fresh orange juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

6.4.3 Protection of probiotic bacteria in pomegranate juice

Similar to orange juice, tests were also performed with pomegranate juice to determine the effect of γ-PGA on the viability of probiotic bacteria during juice storage.

6.4.3.1 *B. longum*

The effect of 2.5% γ-PGA was tested on the viability of *B. longum* in pomegranate juice. It was observed that unprotected cells survived for only 4 days with a viability of 4.07 log CFU/ml (**Fig 6.7**). Complete cell death was observed by day 6. In contrast, when the cells were protected with γ-PGA, cell viability of 6.17 log CFU/ml was observed at day 4. Polymer protected cells survived for 13 days with a viability of 3.37 log CFU/ml. Cells lost complete viability within 20 days.

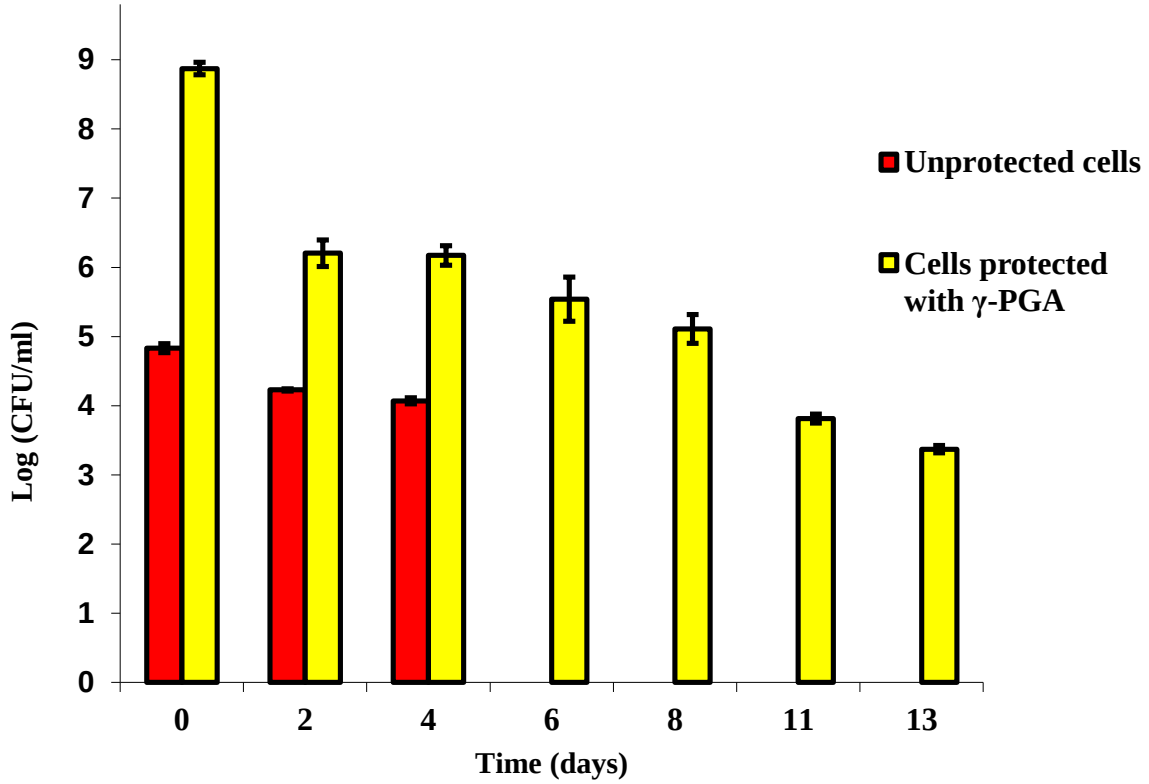


Fig 6.7: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. longum* in pomegranate juice. Unprotected and γ-PGA protected cells were introduced into fresh pomegranate juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

6.4.3.2 *B. breve*

The effect of 2.5% γ-PGA was also tested on the viability of *B. breve* in pomegranate juice. Unprotected cells died within 2 days of storage in pomegranate juice (**Fig 6.8**). On inoculation, the viability of unprotected cells was 5 log CFU/ml. When the cells were protected with γ-PGA, they survived for 11 days, after which complete loss in viability was observed. Even on day 2, viable cell count of γ-PGA-protected cells was 5.13 log CFU/ml, which is below the recommended FAO value of 6-7 log CFU/ml (Kurmann and Rasic, 1991).

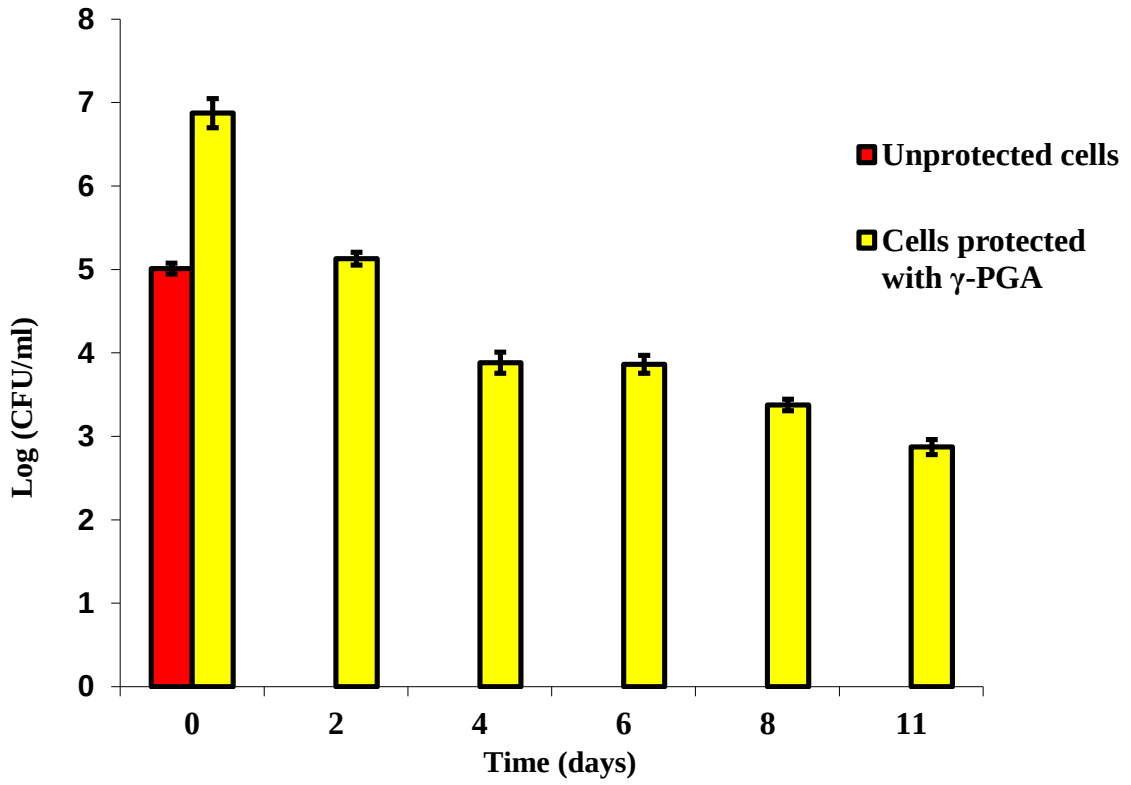


Fig 6.8: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. breve* in pomegranate juice. Unprotected and γ-PGA protected cells were introduced into fresh pomegranate juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

6.4.4 Change in organic acid concentration/pH in orange and pomegranate juice

It was important to identify changes in concentration of organic acids, if any, when probiotic bacteria and γ-PGA are introduced, to preserve the organoleptic properties of the juice. Organic acid analysis was performed using HPLC.

6.4.4.1 *B. longum* in orange juice

It was seen that fresh orange juice contained 425.09 mg/l of ascorbic acid (**Table 6.3**). This concentration dropped to 160.97 mg/l for juice that was stored at 4˚ C for 39 days. When *B. longum* was added to orange juice without γ-PGA, it was seen that the concentration of

ascorbic acid in the juice after 39 days reduced further to 79.14 mg/l. Likewise, when *B. longum* protected with γ-PGA was added to orange juice, the concentration of ascorbic acid after 39 days was 71.52 mg/l.

Table 6.3: Organic acid content of fresh or expired orange juice, with γ-PGA-protected and unprotected *B. longum*. "a" & "b" indicate value is significantly (P < 0.05) and insignificantly (P > 0.05) different from that in expired orange juice, respectively. Experiments were conducted in triplicate (n = 3).

Condition	Ascorbic acid (mg/l)	Citric acid (g/l)	Malic acid (g/l)
Fresh orange juice – No cells	425.09 ± 2.26[a]	9.95 ± 0.02[b]	3.84 ± 0.01[b]
Expired orange juice – No cells after 6 weeks	160.97 ± 35.97	9.75 ± 0.02	3.95 ± 0.01
Unprotected *B. longum* in orange juice after 6 weeks	79.14 ± 14.71[a]	9.19 ± 0.08[b]	4.01 ± 0.15[b]
γ-PGA-protected *B. longum* in orange juice after 6 weeks	71.52 ± 0.61[a]	8.78 ± 0.18[b]	3.63 ± 0.06[b]

The concentrations of citric acid and malic acid in fresh orange juice were 9.95 g/l and 3.84 g/l respectively. After 39 days, the difference in concentration of citric and malic acid in orange juice without *B. longum* was not statistically significant when compared to that of fresh juice (P > 0.05). After 39 days, orange juice with unprotected *B. longum* showed a slightly reduced (9.19 g/l) concentration of citric acid, but this reduction was also not statistically significant (P > 0.05). The citric acid concentration was further reduced slightly (8.78 g/l) for orange juice with γ-PGA-protected cells. The concentration of malic acid in orange juice with unprotected cells and with γ-PGA-protected cells did not differ significantly from that of orange juice without cells after 39 days (P > 0.05).

The pH of fresh orange juice was 3.91 (**Table 6.4**). When *B. longum* protected with γ-PGA were added to the juice, the pH was seen to increase to 4.36 after 39 days.

Table 6.4: pH of fresh or expired orange juice, with γ-PGA-protected and unprotected *B. longum*. "a" indicates value is significantly different from that in expired orange juice (P<0.05). Experiments were conducted in triplicate (n = 3).

Condition	pH
Fresh orange juice – No cells	3.91 ± 0.00
Expired orange juice - after 6 weeks - No cells	3.82 ± 0.00
B. longum in orange juice – No γ-PGA – after 6 weeks	3.71 ± 0.00[a]
B. longum in orange juice – with γ-PGA – after 6 weeks	4.36 ± 0.01[a]

6.4.4.2 *B. breve* in orange juice

When unprotected *B. breve* was added to orange juice, it was seen that the concentration of ascorbic acid in the juice reduced to 173.23 mg/l after 39 days (**Table 6.5**). When *B. breve* protected with γ-PGA was added to orange juice, the concentration of ascorbic acid after 39 days was 193.76 mg/l. The change in ascorbic acid concentration in both conditions was not statistically significant (P > 0.05) when compared to its concentration in orange juice without cells after 39 days (160.97 mg/l).

Orange juice with *B. breve* showed a slightly reduced citric acid concentration (9.29 g/l) after 39 days, but this reduction was statistically insignificant (P > 0.05). The citric acid concentration further reduced slightly (8.31 g/l) for orange juice with γ-PGA protected cells (P > 0.05). The concentration of malic acid in orange juice with unprotected *B. breve* did not significantly differ (P > 0.05) from that of orange juice without cells after 39 days. The malic acid concentration in orange juice with γ-PGA protected cells showed a small change in concentration (0.52 g/l) in comparison to orange juice without any cells and γ-PGA after 39 days.

Table 6.5: Organic acid content of fresh or expired orange juice, with γ-PGA-protected and unprotected *B. breve*. "a" & "b" indicate value is significantly (P<0.05) and insignificantly (P>0.05) different from that in expired orange juice, respectively. Experiments were conducted in triplicate (n = 3).

Condition	Ascorbic acid (mg/l)	Citric acid (g/l)	Malic acid (g/l)
Fresh orange juice - No cells	425.09 ± 2.26[a]	9.95 ± 0.02[b]	3.84 ± 0.01[b]
Expired orange juice - No cells after 6 weeks	160.97 ± 35.97	9.75 ± 0.02	3.95 ± 0.01
Unprotected *B. breve* in orange juice after 6 weeks	173.23 ± 18.48[b]	9.29 ± 0.22[b]	3.92 ± 0.08[b]
γ-PGA-protected *B. breve* in orange juice after 6 weeks	193.76 ± 12.49[b]	8.31 ± 0.02[b]	3.43 ± 0.03[b]

The pH of fresh orange juice was 3.91 (**Table 6.6**). When *B. breve* cells protected with γ-PGA were added to the juice, the pH was seen to increase to 4.22 after 39 days.

Table 6.6: pH of fresh or expired orange juice, with γ-PGA-protected and unprotected *B. breve*. "a" indicates value is significantly different from that in expired orange juice (P<0.05). Experiments were conducted in triplicate (n = 3).

Condition	pH
Fresh orange juice – No cells	3.91 ± 0.00
Expired orange juice - after 6 weeks - No cells	3.82 ± 0.00
B. breve in orange juice – No γ-PGA – after 6 weeks	3.65 ± 0.00[a]
B. breve in orange juice – with γ-PGA – after 6 weeks	4.22 ± 0.02[a]

6.4.4.3 *B. longum* in pomegranate juice

When the change in organic acid concentration for pomegranate juice was assessed (see **Table 6.7**), it was seen that there was no significant difference (P > 0.05) in concentrations of citric acid and malic acid in juice stored for 39 days with or without γ-PGA-protected *B. longum*. The ascorbic acid concentration in fresh pomegranate juice was negligible (9 mg/l). The concentration was seen to increase slightly in juice with γ-PGA protected *B. longum*. However, even in this case, the concentration was low (25.52 mg/l).

Table 6.7: Organic acid content of fresh or expired pomegranate juice, with γ-PGA-protected and unprotected *B. longum*. "a" & "b" indicate value is significantly (P<0.05) and insignificantly (P>0.05) different from that in expired pomegranate juice, respectively. Experiments were conducted in triplicate (n = 3).

Condition	Citric acid (g/l)	Malic acid (g/l)	Ascorbic acid (mg/l)
Fresh pomegranate juice - No cells	17.43 ± 0.48[b]	4.75 ± 0.90[b]	9.00 ± 1.51[b]
Expired pomegranate juice - No cells - after 6 weeks -	17.22 ± 0.51	2.76 ± 0.10	11.39 ± 0.19
Unprotected *B. longum* in pomegranate juice after 6 weeks	19.32 ± 1.10[b]	2.95 ± 0.46[b]	10.96 ± 6.57[a]
γ-PGA-protected *B. longum* in pomegranate juice after 6 weeks	17.84 ± 1.07[b]	3.64 ± 0.19[b]	25.52 ± 0.72[a]

The pH of fresh pomegranate juice was 3.18 (**Table 6.8**). When *B. longum* cells protected with γ-PGA were added to the juice, the pH was seen to increase to 4.15 after 39 days.

Table 6.8: pH of fresh or expired pomegranate juice, with γ-PGA-protected and unprotected *B. longum*. "a" indicates value is significantly different from that in expired pomegranate juice (P<0.05). Experiments were conducted in triplicate (n = 3).

Condition	pH
Fresh pomegranate juice – No cells	3.18 ± 0.00
Expired pomegranate juice - after 6 weeks - No cells	3.19 ± 0.00
B. longum in pomegranate juice – No γ-PGA – after 6 weeks	3.14 ± 0.00
B. longum in pomegranate juice – with γ-PGA – after 6 weeks	4.15 ± 0.06[a]

6.4.4.4 *B. breve* in pomegranate juice

When the change in organic acid concentration for pomegranate juice was assessed (see **Table 6.9**), it was observed that there was no significant difference (P > 0.05) in concentrations of citric acid and malic acid in juice stored for 39 days with or without γ-PGA-protected *B. breve*. The concentration of ascorbic acid in pomegranate juice under various conditions was negligible (≤ 13.31 mg/l).

Table 6.9: Organic acid content of fresh or expired pomegranate juice, with γ-PGA-protected and unprotected *B. breve*. "b" indicates value is insignificantly (P > 0.05) different from that in expired pomegranate juice. Experiments were conducted in triplicate (n = 3).

Condition	Citric acid (g/l)	Malic acid (g/l)	Ascorbic acid (mg/l)
Fresh pomegranate juice – No cells	17.43 ± 0.48[b]	4.75 ± 0.90[b]	9.00 ± 1.51[b]
Expired pomegranate juice - No cells - after 6 weeks	17.22 ± 0.51	2.76 ± 0.10	11.39 ± 0.19
Unprotected *B. breve* in pomegranate juice after 6 weeks	16.10 ± 0.66[b]	4.02 ± 0.07[b]	13.31 ± 2.60[b]
γ-PGA-protected *B. breve* in pomegranate juice after 6 weeks	14.06 ± 0.25[b]	5.23 ± 0.60[b]	4.19 ± 3.65[b]

The pH of fresh pomegranate juice was 3.18 (**Table 6.10**). When *B. breve* cells protected with γ-PGA were added to the juice, the pH was seen to increase to 4.05 after 39 days.

Table 6.10: pH of fresh or expired pomegranate juice, with γ-PGA-protected and unprotected *B. breve*. "a" indicates value is significantly different from that in expired pomegranate juice (P<0.05). Experiments were conducted in triplicate (n = 3).

Condition	pH
Fresh pomegranate juice – No cells	3.18 ± 0.00
Expired pomegranate juice - after 6 weeks - No cells	3.19 ± 0.00
B. breve in pomegranate juice – No γ-PGA – after 6 weeks	3.18 ± 0.00[a]
B. breve in pomegranate juice – with γ-PGA – after 6 weeks	4.05 ± 0.02[a]

6.4.5 Protection of bacteria in simulated gastric juice

A heavy loss in the viability of probiotic bacteria has been seen when they pass through the stomach, which is a region of high acidity (Figueroa-González *et al.*, 2011; Vasiljevic and Shah, 2008). Therefore, it is important to help probiotic bacteria survive better in such an environment. γ-PGA has a unique property of staying stable in a highly acidic environment, where it forms an α-helical structure (Ho *et al.*, 2006a). It also disintegrates in a weaker acidic or neutral pH by conforming to a linear random-coil formation. This property of γ-PGA has not been previously used for protection of probiotic bacteria. After testing the effect of γ-PGA on the viability of *Bifidobacteria* in orange and pomegranate juice, the protective effect of the polymer was tested for *Bifidobacteria* in a simulated gastric juice environment for 4 h.

6.4.5.1 *B. longum*

When the effect of 2.5% γ-PGA was tested on the viability of *B. longum* in simulated gastric juice, it was seen that bacteria protected with the polymer showed a marginal reduction in viability of 0.47 log CFU/ml after 4 h ($P < 0.05$. See **Fig 6.9**). However, unprotected cells showed complete loss in viability within 2 h. Even after an hour in simulated gastric juice, the number of live cells in the absence of γ-PGA (3.96 log CFU/ml) was not significant enough to provide any benefit to the host.

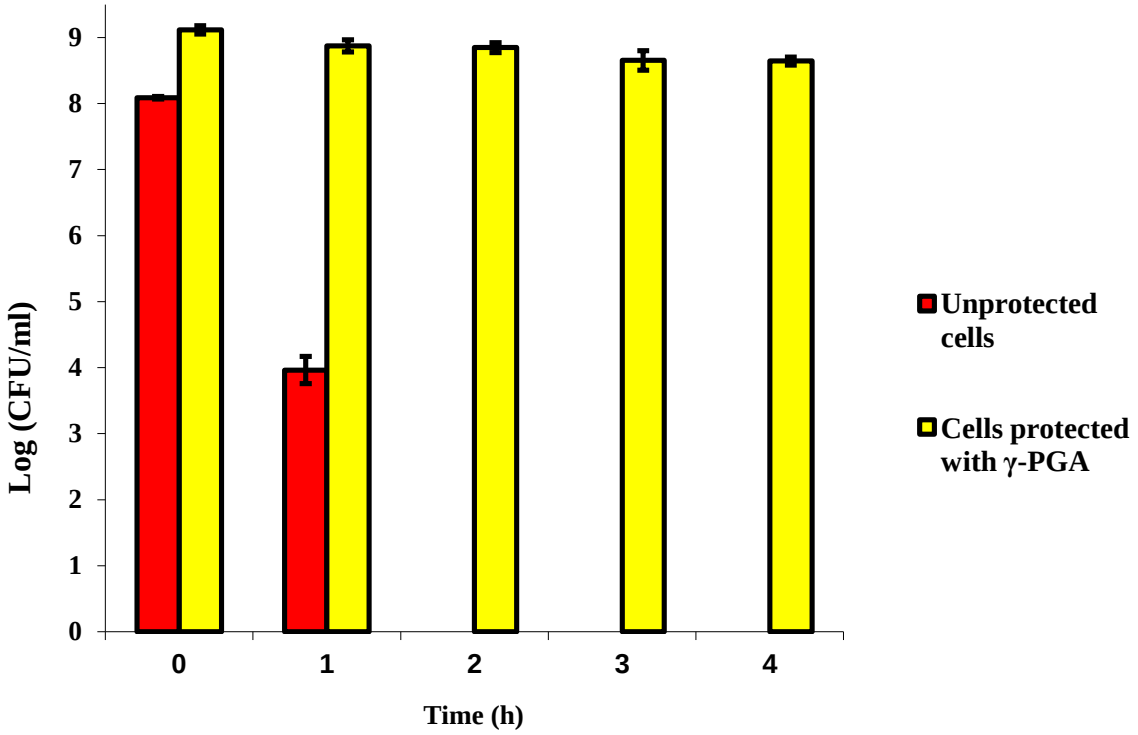


Fig 6.9: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. longum* in simulated gastric juice (pH 2.0). Unprotected and γ-PGA protected cells were introduced into the juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

6.4.5.2 *B. breve*

The effect of 2.5% γ-PGA was also tested on the viability of *B. breve* in simulated gastric juice for 4 h (**Fig 6.10**). *B. breve* protected with the polymer showed even less reduction in viability (0.20 log CFU/ml after 4 h) than *B. longum*. In fact, the number of viable γ-PGA-protected cells after 0 h and 4 h was comparable and was not significantly different ($P > 0.05$). However, unprotected cells showed complete loss in viability within 2 h. Even after an hour in simulated gastric juice, the number of live cells in the absence of γ-PGA (4.22 log CFU/ml) was not significant enough to provide any benefit to the host.

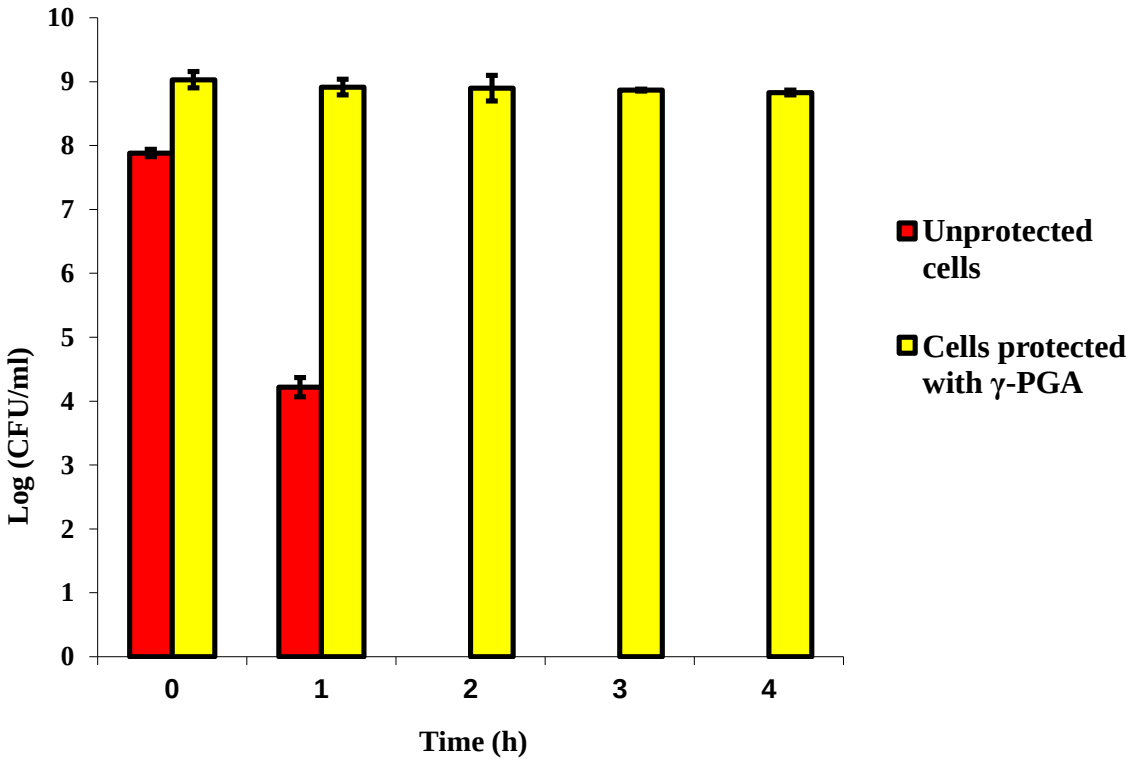


Fig 6.10: Protective effect of 2.5% (w/v) γ-PGA on viability of *B. breve* in simulated gastric juice (pH 2.0). Unprotected and γ-PGA protected cells were introduced into the juice and viability was measured at regular intervals on BSM agar. Experiments were conducted in triplicate (n = 3).

7. DISCUSSION

7.1 Investigation of γ-PGA production in eight bacteria

Eight *Bacillus* strains (*B. subtilis* natto, *B. licheniformis* 9945a, *B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859, *B. licheniformis* 1525 and *B. licheniformis* 6816) were grown in GS and E media to investigate γ-PGA production. Of these bacteria, *B. subtilis* natto and *B. licheniformis* 9945a have been used for γ-PGA production previously (Cromwick *et al.*, 1996; Cromwick and Gross, 1995a; Ho *et al.*, 2006a; Kedia *et al.*, 2010; Shih and Van, 2001; Ko and Gross, 1998), however, the other 6 strains used in this study have not been utilized for this purpose. *B. subtilis* 23856 has been previously used for the production of the volatile hydrocarbon 2-methyl-1,3-butadiene or isoprene while *B. subtilis* 23857 has been used as a source of genomic DNA of mannose-6-phosphate isomerase (Kuzma *et al.*, 1995; Yeom *et al.*, 2009). *B. licheniformis* NCIMB 6816 was used for the production of penicillin (Rao, 1944). After these bacteria were grown in GS and E media, it was observed that all of them produced γ-PGA with various properties (high/low molecular weight, amorphous/crystalline, salt and acid form). Yields of up to 22.3 g/l of γ-PGA in shake flasks were obtained. Therefore, this study contributes six new bacteria for γ-PGA production and helps towards production of more consistent and better quality polymers that are suited to application needs by characterizing the produced γ-PGA.

7.2 Growth and nutrient utilization of *Bacillus* in different media

It is known that bacteria produce γ-PGA in the late logarithmic and stationary phases of growth (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007) and hence, it is important to monitor cell growth in different media. When the eight bacteria were grown in GS medium, it was seen that all bacteria had attained the stationary phase of growth by the first 24 to 48 h (**Fig 4.14**). When grown in medium E, all bacteria (with the exception of *B. licheniformis* 1525, *B. licheniformis* 6816 and *B. subtilis* 23859) took longer to reach

stationary phase of growth as compared to when they were grown in GS medium. This could possibly be due to the presence of a high concentration of glycerol (80 g/l) in medium E, which is known to inhibit cell growth initially (Wu *et al.*, 2010a). It was also evident that each individual bacterial strain reached a higher cell count in GS medium than in medium E. This could be because medium E does not include vitamins in its formulation, whereas GS medium does. *B. subtilis* strains are fastidious in nature (Kedia *et al.,* 2010) and grow to higher numbers in the presence of vitamins. Again, a high concentration of glycerol could also be a reason for lower cell counts in medium E.

γ-PGA production makes the medium viscous which impedes the oxygen mass transfer, thus causing oxygen depletion, hence, controlled agitation and aeration are crucial for optimizing γ-PGA production (Bajaj and Singhal, 2011). None of the strains under study that were grown in shake flasks in GS and E media reached a cell number greater than 10 log CFU/ml. This could probably be due to the lack of pH control/aeration control in shake flasks. Such cell numbers have been achieved when *B. subtilis* natto was grown in fermenters in this study (**Fig 5.**1) and in a previous study when aeration and agitation could be controlled (Kedia *et al.,* 2010).

Analysis of nutrient utilization by HPLC revealed that *B. subtilis* strains consumed more of the L-glutamic acid in GS medium than the *B. licheniformis* strains (**Tables 4.4 & 4.5**). In contrast, *B. licheniformis* strains consumed more L-glutamic acid than *B. subtilis* strains did in medium E. In fact, apart from *B. licheniformis* 6816 and *B. licheniformis* 9945a, which consumed more L-glutamic acid in medium E than when they were grown in GS medium, all bacteria consumed more L-glutamic acid when they were grown in GS medium than when they were grown in medium E. The function of L-glutamic acid in γ-PGA production using

B. subtilis IFO3335 has been described previously (Kunioka and Goto, 1994). It was found that this bacterium does not produce γ-PGA in the absence of exogenously supplied L-glutamic acid. When L-glutamic acid was supplied in the medium, γ-PGA was produced. However, the L-glutamic acid provided was not metabolized by the cells for γ-PGA synthesis. It was concluded that L-glutamic acid added in the medium merely acted as an activator of enzymes responsible for γ-PGA synthesis. In our study, it can be observed that L-glutamic acid was clearly metabolized by the cells (especially by *B. subtilis* strains in GS medium), which indicates that bacteria may have utilized exogenous L-glutamic acid as a substrate for γ-PGA production, as was observed in a previous study (Yao *et al.*, 2010). However, additional tests must be done to confirm this. For classification purposes, γ-PGA producing bacteria have been divided into two groups depending on whether they require L-glutamic acid in the medium and the ones that do not require L-glutamic acid (Bajaj and Singhal, 2011; Buescher and Margaritis, 2007; Shih and Van, 2001; Sung *et al.*, 2005). For L-glutamic acid dependent bacteria, the γ-PGA yield increases with an increase in the L-glutamic acid concentration in the medium. In a follow up study to our work, it was seen that the concentration of γ-PGA produced by the bacteria increased with an increase in the concentration of L-glutamic acid in the medium (data not shown), thus indicating that these γ-PGA producing bacteria were L-glutamic acid dependent.

Important information can be obtained when L-glutamic acid concentration is monitored during cell culture for γ-PGA production. For instance, L-glutamic acid concentration increased after 72 h when *B. subtilis* natto were grown in medium E (**Fig 4.5b**). This could indicate the presence of γ-PGA depolymerase activity. γ-PGA depolymerase is an extracellular enzyme which is active in some bacteria in the late stationary phase and can break down the polymer. This releases L-glutamic acid units, hence causing an increase in the

L-glutamic acid concentration. Degradation of synthesized γ-PGA could be one of the reasons why *B. subtilis* natto produced only 5.7 g/l γ-PGA after 96 h in medium E. It has been shown previously that *B. subtilis* produces γ-PGA degradation enzymes such as GGT, YwtD and PgdS (Ashiuchi *et al.*, 2006; Jeong *et al.*, 2010; Mitsui *et al.*, 2011; Yao *et al.*, 2009). The increase in L-glutamic acid concentration after 72 h in our study could be a consequence of the components of such an enzyme degradation system. Interestingly, L-glutamic acid concentration did not increase after 72 h when *B. subtilis* natto was grown in GS medium. This provides important information on when γ-PGA should be recovered from culture in different media. Presence of γ-PGA depolymerase activity can alter yield and molecular weight of isolated γ-PGA and hence, it is important to recover γ-PGA from culture media before γ-PGA depolymerase becomes active. It would be interesting to recover γ-PGA for *B. subtilis* natto grown in medium E at 72 h, to test whether an increased yield is obtained.

At the end of 96 h in medium E, about 40-55 g/l of glycerol of the 80 g/l provided was consumed by all bacteria in shake flasks with low aeration conditions (**Table 4.5**). Similar results were obtained by Cromwick and co-workers (1996) when the consumption of glycerol was assessed in low aeration conditions for *B. licheniformis* 9945a (Cromwick *et al.*, 1996). In high aeration conditions and at pH 6.5, most of the glycerol was consumed in their study. In another study (Birrer *et al.*, 1994), when *B. licheniformis* 9945a was grown in shake flasks in medium E, it was seen that glycerol and L-glutamic acid were not completely consumed and substantial quantities of these nutrients were still left in the medium after 96 h and only 35 g/l of glycerol and 8 g/l of L-glutamic acid were consumed by bacteria by the end of the culture. A similar observation was made in our study for L-glutamic acid consumption, where *B. licheniformis* 9945a consumed ~ 7 g/l of L-glutamic acid. However, more glycerol was consumed in our study (57 g/l) and this is probably why *B. licheniformis* 9945a produced

more γ-PGA (12.58 g/l) after 96 h in this study than that produced using the same strain (11 g/l) in the study by Birrer and co-workers (1994).

Yao and co-workers (2010) assessed the consumption of glucose in *B. subtilis* NX-2 and found that only a small fraction of γ-PGA repeat units were formed from exogenously supplied glucose and most of the glucose was used as the growth-limiting substrate for cell growth (Yao *et al.,* 2010). It was noted that for all strains in our study (except *B. subtilis* 23858 and *B. subtilis* 23859), the sucrose concentration remained stable after 72 h (**Figs 4.1 to 4.7**). This could indicate that sucrose could not be transported inside the cells once they were in death phase. In contrast, glycerol concentration was seen to decrease after 72 h for all bacteria (except *B. subtilis* 23857 and *B. subtilis* natto) and hence it can be stated that glycerol transport inside the cells continued even after they were in death phase. It has been shown previously that glycerol is responsible for increasing the permeability of the cell membrane, thus improving the transport of γ-PGA outside the cell (Bajaj and Singhal, 2011; Wu *et al.*, 2010a). This could be one of the reasons for utilization of glycerol even after cell death commenced in culture.

Overall, it can be seen (**Tables 4.4 & 4.5**) that all of the *B. subtilis* strains (except *B. subtilis* 23859) consumed more sucrose (in GS medium) than glycerol (in medium E). Sucrose has been preferred to other C sources for γ-PGA production in *B. subtilis* previously (Ashiuchi and Misono, 2002; Kedia *et al.*, 2010). On the other hand, *B. licheniformis* consumed more glycerol (in medium E) than sucrose (in GS medium) in our study. Improved yield of γ-PGA using *B. licheniformis* has been obtained when glycerol was used as the C source in a previous study (Du *et al.*, 2005). In terms of L-glutamic acid consumption, all of the *B. subtilis* strains consumed more L-glutamic acid when grown in GS medium than in medium

E, whereas it was utilized more by all of the *B. licheniformis* strains (except *B. licheniformis* 1525) grown in medium E. Utilization of nutrients has been compared for bacteria grown under different pH, aeration and agitation conditions (Cromwick *et al.*, 1996; Wu *et al.*, 2010b). In our study, the utilization of sucrose, glycerol and L-glutamic acid in two media by *B. subtilis* and *B. licheniformis* has been compared and adds to the existing knowledge on γ-PGA production.

Since the initial experiments were performed in shake flasks, where pH and aeration of the culture were not controlled, it could have some impact on nutrient consumption. The initial pH for both media (7.2) decreased to ~ 6.5 after 30 h, but increased to ~ 6.8 at the end of 96 h. The decrease in pH is believed to be due to acid formation (Thorne *et al.*, 1954). The agitation during production of γ-PGA using bacteria in shake flask cultures (where the volume ratio of culture to flask was 1:2) was controlled at 150 rpm. Cromwick *et al.* (1996) have previously highlighted the importance of pH and aeration for nutrient utilization in *B. licheniformis* 9945a (Cromwick *et al.*, 1996). Their study showed that bacteria utilized glutamate, glycerol and citric acid maximally when the pH was maintained at 6.5 under high aeration conditions (agitation speed 800 rpm, aeration rate 2.0 L/min). The yield obtained under such conditions was much better (23 g/l) than when bacteria were grown in low aeration and agitation conditions in their study (6.3 g/l). In another study, it was found that a pH of 7.0 was optimum for γ-PGA production from *B. subtilis* IFO3335 (Richard and Margaritis, 2003a). In some cases, a two stage pH shift strategy needs to be employed when the optimal pH for growth and γ-PGA production are different. For instance, when γ-PGA production in *B. subtilis* CGMCC 0833 was analysed (Wu *et al.*, 2010b), it was found that the optimum pH for glutamate utilization was 6.5, whereas the optimum pH for cell growth was found to be 7.0. Consequently, a two stage pH shift strategy was proposed, in which the pH

was kept at 7.0 for 24 h for maximum cell growth, followed by a pH shift to 6.5 for maximum utilization of glutamate. Hence, it is evident that control of pH is important for γ-PGA production and the optimum pH depends on the strain of bacteria used.

After extracting the polymer in the form of a dry powder, it was weighed to calculate the yield of γ-PGA produced. The yield obtained by different bacteria in GS and E media was compared (**Fig 4.9**). Even though the C source and L-glutamic acid were not as fully utilized in medium E (**Tables 4.4 & 4.5**), three of the eight bacterial strains (*B. subtilis* 23857, *B. subtilis* 23859 and *B. licheniformis* 1525) produced significantly more γ-PGA in E medium ($P < 0.05$). Interestingly, while all bacteria reached a higher cell count in GS medium, they produced more γ-PGA/cell in medium E. This could indicate that presence of sucrose (GS medium) improved cell growth, but glycerol (medium E) was better for γ-PGA production. Glycerol has been shown to increase the yield of γ-PGA production previously, by improving the permeability of the cell membrane and hence facilitating the secretion of γ-PGA into the medium of production (Bajaj and Singhal, 2011; Du *et al.*, 2005; Wu *et al.*, 2010a; Jeong *et al.*, 2010). In addition, presence of glycerol is known to increase the amount of glutamic acid formed by increasing the flux from 2-oxoglutarate to glutamic acid (Wu *et al.*, 2008). Although increase in glycerol concentration in the medium has been shown to improve γ-PGA concentration in cell broth, it does not necessarily increase cell growth. This could be the reason why bacteria in our study could produce more γ-PGA in medium E, even though they reached a higher cell number in GS medium. Also, glycerol is known to reduce medium viscosity and hence improve utilization of other nutrients by the cells. However, a recent study showed that a very high concentration of glycerol (80 g/l), such as that used in our study, inhibits cell growth of *B. subtilis* NX-2 and hence it has been suggested that glycerol could be added at ~16 h to reduce the initial inhibition of cell growth (Wu *et al.*, 2010a). Our

investigations were performed before this study was published. However, the effects of glycerol and its consumption would differ with the bacterial strain used for γ-PGA production and would need to be investigated. The presence of citric acid in medium E could also explain the increased production of γ-PGA/cell in this medium. Citric acid is the best precursor for production of γ-PGA (Du *et al.*, 2005; Xu *et al.*, 2005a) and can be used by the cells for the production of endogenous L-glutamic acid which is then incorporated into the growing γ-PGA chain (see **Section 1.4.2**). Ammonium ions, which are necessary for the endogenous production of glutamic acid from citric acid, are also present in medium E (Kunioka and Goto, 1994).

The only strain that produced significantly more γ-PGA ($P < 0.05$) in GS medium than in medium E was *B. subtilis* natto (**Fig 4.9**). It also consumed ~95% of the L-glutamic acid present. GS medium was obviously more suited for growth and γ-PGA production by *B. subtilis* natto. This is in accordance with the findings of Kedia and co-workers (Kedia *et al.*, 2010). Our investigation with eight strains indicates that nutrient consumption alone cannot provide an indication of the yield of γ-PGA produced. Yield is dependent on bacterial strain, media components and how efficiently the available nutrients are converted into γ-PGA by the bacteria.

B. licheniformis 9945a has been previously investigated for γ-PGA production using medium E (Cromwick *et al.*, 1996). When the bacteria were grown in fermenters, where pH could be controlled at 6.5, the yield of γ-PGA obtained after 96 h was 15 g/l. When these bacteria were grown in medium E for our study, yield of γ-PGA obtained after 96 h was 12.58 g/l. The lower yield was expected because bacteria were grown in shake flasks, where pH, aeration and agitation could not be controlled. Whilst we could have investigated production of γ-

PGA using all strains in fermenters, the aim of our study was to characterize and compare γ-PGA produced by a range of bacteria in two media. Therefore, investigation experiments were performed in shake flasks. However, it must be noted that γ-PGA yield obtained using *B. licheniformis* 9945a in our study was comparable to that achieved by Kedia and co-workers (Kedia *et al.*, 2010).

When production of γ-PGA by *B. licheniformis* WBL-3 was optimized using different concentrations of glycerol (0 to 110 g/l) in modified medium E in shake flasks, a maximum yield of 16.7 g/l was obtained (Du *et al.*, 2005). Four of the eight strains in our study (*B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859 and *B. licheniformis* 1525) produced γ-PGA with yields higher than 19 g/l in medium E without any optimization (**Fig 4.9**). It would be worth optimizing the production of γ-PGA with respect to carbon source and nitrogen source for these bacteria within fermenters.

7.3 Biopolymer characterization

The polymer produced by all bacteria in GS medium was white in colour. When medium E was used to grow cells, a brownish product was obtained, possibly due to the presence of residual ferric ions (0.2 g/l $FeCl_3 \cdot 6H_2O$). It must be noted that the colour for γ-PGA obtained using medium E is white when precipitated. However, it changes to brown on exposure to oxygen when it is dried. The colour of the polymer produced is important for some applications. A white product, such as that produced by cells in GS medium, would be ideal for applications in the cosmetic and food industries. On the other hand, a brownish polymer, such as that produced in medium E, could be used in its natural form in the waste water treatment industry.

It is evident that all bacteria produced γ-PGA in both media, as confirmed by FT-IR spectroscopy (**Figs 4.10 & 4.11**) and spectra for γ-PGA obtained from all strains in both media compared well to that of a commercially available γ-PGA sample. Ho and co-workers (2006a) have highlighted the characteristic peaks present in the IR spectra of γ-PGA (Ho *et al.*, 2006a).Whilst the spectra obtained in our studies are similar to those of Ho *et al.* (2006a), some differences were noted. The FT-IR spectra for γ-PGA samples produced by all bacteria and that of the commercial sample we used for comparison showed a distinct peak between 820 - 850 cm^{-1}, which was not seen in the spectra produced by Ho and co-workers. This peak represents C-H bending and could further help in the identification of γ-PGA. Overall, the FT-IR spectra for γ-PGA produced in our study in both media showed distinct peaks at the characteristic wavelengths described in the work of Ho *et al* (2006a).

Solubility of γ-PGA is an important parameter with respect to its application. For example, the hydrophilic insoluble form of γ-PGA can be used for water absorption in diapers (Qu and Varennes, 2009; Ho *et al.*, 2008). The free acid form of γ-PGA is insoluble in water and is only soluble in the organic solvent DMSO (Ho *et al.*, 2006a). On the other hand, the salt forms of γ-PGA are completely soluble in water. In addition, crystallinity of the polymer can also affect its solubility in water. γ-PGA produced using eight bacteria in both media was analysed for crystallinity and form (acid/salt) using XRD and ICP-AES respectively (**Figs 4.12 & 4.13**). The results shown in **Sections 4.5 & 4.6** indicate that the production medium has an impact on the form (acid or salt form of γ-PGA) and crystallinity of γ-PGA produced, independent of the bacterial strain used for production. When GS medium was used, all *B. subtilis* and *B. licheniformis* strains produced the salt form of γ-PGA (majorly being sodium salt; see **Table 4.1**), possibly due to the presence of a high concentration (50 g/l) of NaCl in the medium. However, in medium E, the acid form was also obtained along with the sodium

salt form. The production of sodium salt form of γ-PGA in medium E (which does not originally contain sodium) could be explained due to addition of 3M NaOH for adjustment of pH. It would be interesting to exclude sodium completely from medium E by using NH_4OH instead of NaOH for adjustment of pH to determine whether the bacteria then produce a pure acid form of the polymer.

Likewise, bacteria grown in GS medium produced a crystalline polymer, whereas they produced an amorphous product in medium E. As mentioned above, bacteria produced the sodium salt of γ-PGA in GS medium. The presence of sodium ions in the polymer could possibly reduce electrostatic repulsion and hence encourage the formation of crystal lattices. When the orientation of the component L- and/or D-glutamic acid residues in γ-PGA follows a uniform pattern, a crystalline polymer is produced. Conversely, an amorphous polymer would be produced when the orientation of the enantiomers is random. The racemase enzyme (responsible for conversion of L-glutamic acid to D-glutamic acid) might be regulated differently by the components of GS and E media, thus having an effect on crystallinity of γ-PGA. This would warrant further investigation since the factors affecting the crystallinity of γ-PGA have not been previously reported.

The eight bacterial strains produced γ-PGA with different forms and crystallinity, which could have multiple applications depending on requirement of solubility and form of the polymer. Although a lot of research has been done to assess the production and properties of γ-PGA, not much attention has been given to crystallinity. Similar to characterizing the form of γ-PGA, knowledge of the crystallinity of the product could also determine its application.

It is known that γ-PGA can be composed of only L, or only D, or both L and D forms of glutamic acid in varying proportions (Buescher and Margaritis, 2007). A mechanism for racemization of L-glutamic acid to D-glutamic acid (**Fig 1.3**) has been proposed and has been explained by multiple researchers (Ho *et al.*, 2006a; Shih and Van, 2001). Upon racemization, the L-glutamic acid and D-glutamic acid are incorporated into the growing γ-PGA chain. It has also been found that when γ-PGA is composed of only L or only D glutamic acid residues, it is soluble in ethanol (Candela and Fouet, 2006). When it has an equimolar proportion of both D and L subunits, it precipitates in ethanol. Since in this study, the γ-PGA obtained from all bacterial strains precipitated in ethanol, it can be concluded that it contained both L and D isomers of glutamic acid, probably mixed in equimolar amounts This would be in accordance with the information provided by Ho and co-workers (2006), which stated that γ-PGA produced by *B. subtilis* natto contained both L (48%) and D (52%) enantiomers of glutamic acid (Ho *et al.*, 2006a). However, the exact D/L composition of γ-PGA produced by bacteria in our study needs to be analysed.

Molecular weight could be affected by how many glutamic acid residues bacteria are able to polymerize before transporting it outside the cell, presence of extracellular depolymerase activity and nutrients available in the growth medium. The molecular weight of γ-PGA produced in our study ranged from 3000 Da to 871,000 Da and was dependent on both bacterial strain and media used (**Tables 4.2, 4.3, 4.6 & 4.7**). Although *B. licheniformis* 6816 and *B. subtilis* natto produced a high molecular weight polymer in both media, *B. licheniformis* 1525 produced a high molecular weight polymer in GS medium alone. All other bacteria produced γ-PGA with a low molecular weight (~3000 Da).

Molecular weight plays an important role during optimization of γ-PGA for an application. γ-PGA with different molecular weights could be used for different applications. For instance, molecular weight has been found to be a decisive factor for drug delivery applications of γ-PGA (Buescher and Margaritis, 2007). Polymers of different molecular weights are required to control the drug's release into the tissue. Low molecular weight γ-PGA has been shown to exhibit a high antifreeze activity (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003). In contrast, Kim and co-workers have shown that administering γ-PGA with a molecular weight as high as 2 x 10^6 Da orally, induces significant Natural Killer (NK) cell-mediated antitumour immunity in mice bearing MHC class I-deficient tumours (Kim *et al.*, 2007). The antimutagenic properties of γ-PGA itself have been shown to be dependent on molecular weight, where γ-PGA with molecular weights of greater than 5 x 10^4 Da and lower than 6 x 10^6 Da showed distinct antimutagenic property. γ-PGA with a molecular weight of 9.9 x 10^5 Da has been shown to be effective for waste water treatment applications (Inbaraj *et al.*, 2006). In addition, a study (Sung *et al.*, 2005) has indicated that molecular weight of γ-PGA has an effect on calcium solubility, where the soluble calcium uptake in mice was significantly higher when they were administered γ-PGA with a higher molecular weight (5 x 10^6 Da) than γ-PGA with a lower molecular weight (1 x 10^6 Da).

7.4 Importance of characterizing γ-PGA from different bacterial strains

There has been a lot of attention paid to the application of γ-PGA for use in the field of medicine, food, cosmetics, waste water treatment etc. (Hsieh *et al.*, 2005; Izumi *et al.*, 2007; Mitsuiki *et al.*, 1998; Octavio *et al.*, 2012; Sonaje *et al.*, 2010; Sung *et al.*, 2007; Tanimoto *et al.*, 2007; Tsao *et al.*, 2011; Uotani *et al.*, 2011; Ye *et al.*, 2006). However, characterisation of γ-PGA and identification of the factors affecting γ-PGA characteristics have not been covered in as much detail. An understanding of these characteristics is important to produce a better

quality polymer with more consistency. The γ-PGA produced using the eight strains in this study, six of which (*B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859, *B. licheniformis* 1525 & *B. licheniformis* 6816) having not previously been used for γ-PGA production, was characterized with respect to crystallinity, form and molecular weight. It was found that crystallinity and form of the polymer are dependent on medium of production and not on the bacterial strain. On the other hand, yield and molecular weight of γ-PGA produced in our study were dependent on both bacteria and medium used for production. γ-PGA with yields (up to 22.3 g/l) and different properties produced in this study could not only be used for current applications, but also for some potential novel applications such as maintaining probiotic viability under different conditions. Molecular weight, crystallinity and form of γ-PGA affect its solubility, which in turn would affect its application.

As mentioned in **section 7.3**, low molecular weight γ-PGA produced in this study is especially important for drug delivery applications. Researchers often have to resort to molecular weight reduction techniques for γ-PGA applications that require a low molecular weight polymer (Shih and Van, 2001). In our research, bacteria have produced low molecular weight polymer (~3000 Da), which could be used directly for such applications. This would make the extra step of molecular weight reduction unnecessary, thus making the process more time and cost efficient. On the other hand, high molecular weight γ-PGA can be used for other applications such as waste water treatment. γ-PGA possesses the unique property of remaining stable in low pH environments and disintegrating in a near neutral environment, which can be exploited for novel functional food applications.

7.5 Large scale production of γ-PGA by *B. subtilis* natto

After identification and characterization of the polymer produced by eight bacteria in both media, γ-PGA produced by *B. subtilis* natto in GS medium was chosen for probiotic tests for the following reasons. Firstly, γ-PGA produced by this bacterium is ideal for food applications. *B. subtilis* natto is food derived since it was isolated from a traditional Japanese food item called natto. In GS medium, all strains tested produced Na-γ-PGA (**Table 4.6**), which is known to have good antifreeze activity (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003) and could serve as a cryoprotectant for maintaining viability of probiotic bacteria when they are freeze dried. In addition, Na-γ-PGA is tasteless and hence can be added to food products without considerably changing their taste (Ho *et al.*, 2006a). Also, due to the existence of strong intra-molecular hydrogen bonding, Na-γ-PGA is known to conform to a stable and compact α-helical structure in an environment with a low pH (Ho *et al.*, 2006a). In contrast, at a higher pH, the intra-molecular hydrogen bonding is disrupted, thus disintegrating the polymer into a random coil conformation. This property of Na-γ-PGA would be useful to protect probiotic bacteria in the stomach, which is a region of high acidity (pH 2.0). Also, once Na-γ-PGA reaches the intestine, which is a region of weak acidic-neutral pH, the polymer should disintegrate because of the disruption of hydrogen bonds and release the bacteria into the target site. Moreover, γ-PGA produced in GS medium is white in colour which is preferred for a food application. Compared to other bacteria under study, *B. subtilis* natto produced the highest yield of γ-PGA (17.77 g/l) in GS medium (**Table 4.6**), and hence, it would produce the highest quantity of γ-PGA for probiotic tests.

B. subtilis natto, produced a very high molecular weight polymer in medium E (**Table 4.7**). A high molecular weight polymer would have a high water absorption capability and would hence form hydrogels when introduced in food. This would change the consistency of the

food product, which is undesirable. It has been shown previously that γ-PGA with a molecular weight of less than 20,000 Da has excellent antifreeze activity (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003). In GS medium, γ-PGA with a molecular weight of 257,000 Da was produced (**Table 4.6**). The molecular weight of this γ-PGA could be reduced using a simple aqueous hydrolysis technique (Goto and Kunioka, 1992). Therefore, γ-PGA with a moderate molecular weight, such as the one produced by *B. subtilis* natto in GS medium, was deemed to be more appropriate for the probiotic application.

It was important to scale up the production of γ-PGA using *B. subtilis* natto from 250 ml shake flasks to 4 l fermentations for increasing the yield of γ-PGA obtained through growth in a controlled fermenter environment, and to increase the total quantity of γ-PGA to test the novel probiotic applications.

When *B. subtilis* natto were grown in GS medium in shake flasks, a maximum cell count of 9.03 log CFU/ml was achieved (**Table 4.4**). However, when grown in a fermenter, a cell count of 10.11 log CFU/ml was attained (**Fig 5.1**). This was probably because parameters known to affect the cell growth and production of γ-PGA, such as pH, temperature, aeration and agitation could be controlled and maintained to their optimum levels in the fermenter. γ-PGA production makes the medium viscous which hampers the mass transfer rate of dO_2, thus making it extremely important to ensure that reduction in the saturation percentage of dO_2 does not hamper cell growth. The saturation percentage of dO_2 was maintained above 40% throughout the fermentation of *B. subtilis* natto by automatically controlling the agitation and aeration. Increased biomass and optimized growth conditions could be the reason why a significantly higher ($P < 0.05$) yield of γ-PGA was obtained in fermenters (27.94 g/l) than in shake flasks (17.7 g/l). The yield of γ-PGA from *B. subtilis* natto grown in

fermenters in this study is comparable to that achieved by previous researchers (Ho *et al.*, 2006a; Kedia *et al.*, 2010).

γ-PGA produced by *B. subtilis* natto in a 4 l fermentation was analysed by FT-IR spectroscopy and spectra for both crude and pure (dialyzed) γ-PGA were compared (**Fig 5.4**). γ-PGA that was purified using dialysis showed additional peaks at 1641 cm^{-1} and 1236 cm^{-1}, which correspond to amide I N-H bending and C-O stretch respectively. Also, ICP-AES analysis showed that pure γ-PGA had a lower concentration of sodium and potassium ions than the crude polymer. This indicates that the presence of lower molecular weight γ-PGA and salt impurities in crude γ-PGA can be eliminated by dialysis. This is the first report that demonstrates the difference between characteristics of crude and pure γ-PGA.

7.6 γ-PGA sterilization

According to our knowledge, this is the first report on sterilization of γ-PGA available in the literature. Whilst γ-irradiation of γ-PGA could work on a large scale, it is too costly when smaller amounts of γ-PGA need to be sterilized. In addition, irradiation would result in cross-linking of γ-PGA, thus increasing its water absorption capability (Sung *et al.*, 2005). A polymer with a high water absorption capability would form hydrogels in solution. This is not ideal since it would change the palatability of the foodstuff in which it is to be used.

Different techniques were tested for the sterilization of γ-PGA (**Table 6.1**) and of these, autoclaving at 0.35 BAR & 110˚C for 30 min, autoclaving at 1.035 BAR & 121˚C for 20 mins and treatment with 3% hydrogen peroxide were effective for γ-PGA sterilization. These techniques could completely eradicate the residual bacteria and their spores. Obviously, autoclaving γ-PGA at 0.35 BAR & 110˚C for 30 mins would expose it to less stressful

conditions than autoclaving it at 1.035 BAR & 121˚C for 20 mins. Between autoclaving at 0.35 BAR & 110˚C for 30 mins and treatment with 3% hydrogen peroxide, autoclaving was preferred for sterilization of γ-PGA produced in our study. This is because from FT-IR analysis, it was evident that autoclaving did not affect the structural integrity of γ-PGA, whereas the spectrum of γ-PGA treated with 3% hydrogen peroxide indicated changes in the amide II stretch and C=O symmetric stretch regions when compared to the spectrum for untreated γ-PGA (**Fig 6.1**). This could mean that 3% hydrogen peroxide treatment affects the structure of the polymer. In addition, it is possible that any traces of hydrogen peroxide that may be left after recovery of sterilized γ-PGA could affect the subsequent viability of probiotic bacteria to be tested. Moreover, although hydrogen peroxide is routinely used for sterilization of foodstuffs such as salads, there could be palatability and odour issues.

Results presented here thus indicate that steam sterilization by autoclaving did not seem to affect the structure of γ-PGA. A previous study (Ho *et al.*, 2006a) has reported that the decomposition temperature of Na-γ-PGA is 340˚C. Therefore, heating γ-PGA at 110˚C would not disrupt its structure. An added advantage of heating up the polymer in an aqueous solution is manipulation of molecular weight. When Goto and Kunioka (1992) performed aqueous hydrolysis of γ-PGA at 100˚C, the molecular weight of γ-PGA reduced from 226 KDa to 117 KDa in 30 mins, whilst heating an aqueous solution of the polymer at 120˚C for 30 mins reduced the molecular weight from 226 KDa to 18 KDa (Goto and Kunioka, 1992). The authors also mention that the activation energy of the polymer chain scission due to the hydrolysis of γ-PGA by heating is approximately 120 kJ/M. The relative bond strengths of the C-C, C-N and C-O bonds are greater than 300 kJ/M. Hence, the breaking of the bonds by heating at 120˚C is unexpected. In our study, an aqueous solution of γ-PGA with a molecular weight of 257 KDa was heated at 110˚C for 30 mins at 0.35 BAR Therefore, it can be said

that although using this technique for sterilization of γ-PGA did not affect the structural bonds within the polymer, it reduced its molecular weight below 257 KDa (possibly between 20 and 133 KDa). It has been shown previously that γ-PGA with a lower molecular weight (~20 KDa) has a better antifreeze activity than a higher molecular weight γ-PGA (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003). Therefore, autoclaving γ-PGA could not only sterilize the polymer, but might also improve its efficiency in protecting the cells when they are freeze dried (see **Section 7.7**). The reduction in molecular weight of γ-PGA when it is autoclaved needs to be assessed using GPC.

7.7 Cryoprotection of probiotic bacteria with γ-PGA

Although the antifreeze activity of γ-PGA has been assessed previously (Mitsuiki *et al.*, 1998; Mizuno *et al.*, 1997; Shih *et al.*, 2003), it has never been used to maintain and protect the viability of bacteria. This study is the first to assess the effect of γ-PGA on the viability of probiotic bacteria when they are freeze dried.

Since autoclaving would reduce the molecular weight of the polymer, it was essential to determine whether sterilizing γ-PGA changed its ability to protect the cells during freeze drying. Therefore, γ-PGA sterilized by autoclaving (γ-PGA[S]) and untreated γ-PGA (γ-PGA[U]) were tested as cryoprotectants for *L. paracasei*, *B. breve* and *B. longum*. A non-selective medium, TPY agar, was used for enumeration of cells for tests with γ-PGA[S]. A selective and differential medium for *Bifidobacteria* (BSM agar) was used for tests with γ-PGA[U] so that the residual bacteria in γ-PGA[U] would not interfere with subsequent viability counts of probiotic bacteria. When 22 h and 16 h cultures of *B. breve* and *B. longum* were plated on TPY and BSM agar, it was seen that the counts on both media were not

significantly different ($P > 0.05$. See **Table 6.2**). Hence, the results obtained for the freeze drying experiments using TPY and BSM could be compared.

When unprotected bacteria, or bacteria protected with γ-PGA[S], or sucrose were freeze dried (**Fig 6.2**), it was seen that *L. paracasei* was more resistant to the freeze drying process than both the *Bifidobacteria* strains under study. There was 1.34 log CFU/ml reduction in viability of *L. paracasei* when they were freeze dried without a cryoprotectant (i.e. unprotected cells). For the *Bifidobacteria* strains, 2.47-2.52 log CFU/ml reduction in viability was observed for unprotected cells.

The results for freeze drying with γ-PGA as a cryoprotectant showed that for *L. paracasei*, 10% γ-PGA[S] could protect the cells during freeze drying significantly better than 10% sucrose ($P < 0.05$). Although 5% γ-PGA[S] was also able to protect the cells during freeze drying, it was not as efficient as 10% γ-PGA[S]. However, its cryoprotectant ability was comparable to 10% sucrose ($P > 0.05$). For *B. longum* and *B. breve*, 10% γ-PGA[S] and 10% sucrose were equally efficient in maintaining viability during freeze drying ($P > 0.05$). 5% γ-PGA[S] was able to protect the cells during freeze drying, but was not as effective as 10% γ-PGA[S] or 10% sucrose. The effect of 20% γ-PGA[S] was also tested. However, the solution was too viscous and was difficult to manipulate. Because of this, the results obtained were inconsistent and are not discussed here.

When γ-PGA[U] was tested for its effect on the viability of probiotic bacteria during freeze drying, it was seen that it could protect the cells when compared to cells that were freeze dried without any cryoprotectant (**Fig 6.3**). However, compared to γ-PGA[S], it was not as effective. This could be due to the fact that heating γ-PGA in an aqueous solution at 110˚C

for 30 min reduces its molecular weight. Therefore, it is evident that a lower molecular weight γ-PGA is a better cryoprotectant than a high molecular weight γ-PGA. This is in accordance with work done previously to determine the antifreeze activity of γ-PGA (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003).

It has been found that sucrose offers better protection during freeze drying of *Lactobacilli* when compared to trehalose and sorbitol (Siaterlis *et al.*, 2009). Since γ-PGA[S] could protect *Lactobacilli* better than sucrose in our study, it could indicate that γ-PGA[S] is a better cryoprotectant than trehalose and sorbitol as well. *Nata* (bacterial cellulose produced by *Acetobacter xylinum*) has also been used to protect different *Lactobacilli* during freeze drying in a study (Jagannath *et al.*, 2010). When the cells were immobilized using *nata* and freeze dried, it was observed that viable cell number decreased (from $10^9 – 10^{10}$ CFU/g to 10^7 CFU/g). In our study, there was only a 0.51 log CFU/ml reduction in viability of γ-PGA[S]-protected *Lactobacilli* (see **Fig 6.2**), hence indicating that γ-PGA may be a better cryoprotectant compared to *nata*. The protection offered by γ-PGA, trehalose, sorbitol and *nata* during freeze drying of *Lactobacilli* needs to be compared by performing additional tests under identical conditions.

On assessing the results obtained in this study, it was evident that 10% γ-PGA[S] can be used as an effective cryoprotectant for probiotic bacteria when they are freeze dried. SEM analysis suggested that probiotic bacteria may be protected by a dual mechanism (**Fig 6.4**). The bacteria appeared to be embedded into the γ-PGA matrix as well as covered with a thin coating of γ-PGA. This afforded them protection against factors that were detrimental to their viability during freeze drying. Additional tests with TEM need to be performed to confirm this observation.

7.8 γ-PGA protection of probiotic bacteria in juice

Fruit juices are good candidates for incorporation of probiotic bacteria, since they are rich in nutrients (Ding and Shah, 2008). Also, fruit juices often have oxygen scavenging agents, such as ascorbic acid, which promote anaerobic growth conditions. In addition, fruit juices contain high amounts of sugars which could encourage growth of the added probiotic bacteria. However, it is often seen that probiotic viability in fruit juice is hampered due to factors such as pH, storage temperature, oxygen levels and presence of competing microorganisms (Granato *et al.*, 2010). Once it was confirmed that γ-PGA[S] could be used as a cryoprotectant for probiotic bacteria, its effect was tested on the viability of probiotic bacteria when stored in 2 fruit juices (orange and pomegranate) for 6 weeks. As mentioned previously (see **Section 1.7.2)**, γ-PGA can be used as a food additive since it is Generally Recognized As Safe (GRAS) by the FDA (Shyu and Sung, 2010).

Because it is known that *Bifidobacteria* are very sensitive to pH conditions (Cui *et al.*, 2000) and our cryoprotectant studies showed that *L. paracasei* was more resistant in enduring the freeze drying process than the *Bifidobacteria* strains under study, the juice experiments were performed with *B. breve* and *B. longum*.

It was best to use a selective and differential medium, such as BSM agar, for enumeration of bacteria for these tests. This is because even though some bacteria are killed during the pasteurization process, the juice is not completely sterile. This was tested by plating some orange juice on TSA. As expected, bacterial growth was observed on the plates after 24 h (**Section 6.4.1**). However, when orange and pomegranate juice were plated on BSM agar, no bacterial growth was observed, which meant that the residual bacteria in the juice would not interfere with the viability counts for subsequent probiotic tests.

The final concentration of γ-PGA used for the juice experiments was 2.5% (w/v). The effect of γ-PGA was tested on the viability of both *Bifidobacteria* strains stored in orange juice for 39 days at 4˚C (**Figs 6.5 & 6.6**). For both bacteria, cells that were not protected with the polymer (unprotected cells), showed complete loss in viability within 20 days. Even on day 11, the number of unprotected cells (< 5 log CFU/ml) was much less than the recommended FAO value of 6-7 log CFU/ml (Kailasapathy and Chin, 2000; Kurmann and Rasic, 1991). Cells that were protected with γ-PGA survived well for 39 days in orange juice. *B. longum* showed a viability of 6.48 log CFU/ml on day 39, whereas *B. breve* showed a viability of 6.61 log CFU/ml. This proves that, if orange juice were to be used as a delivery agent for *Bifidobacteria*, protecting the cells with γ-PGA could increase the shelf life of the probiotic product three times or more, when compared to orange juice with unprotected bacteria. *B. breve* (reduction in viability of 2.05 log CFU/ml) showed better survival in orange juice when protected by γ-PGA than *B. longum* (reduction in viability of 2.99 log CFU/ml) after 39 days.

When the viability of *B. longum* encapsulated in Ca-alginate beads in orange juice was monitored in a study (Ding and Shah, 2008) for a period of 6 weeks, a reduction of ~3.0 log CFU/ml in viability was observed at the end of storage at 4˚C. γ-PGA-protected cells in our study also showed similar loss in viability (2.99 log CFU/ml) over 39 days. However, the study by Ding and Shah (2008) does not highlight the viability of Ca-alginate encapsulated cells in simulated gastric juice environments. It is known from other studies (Anal and Singh, 2007; Chandramouli *et al.*, 2004; Hansen *et al.*, 2002; Krasaekoopt *et al.*, 2004; Mortazavian *et al.*, 2008; Lee and Heo, 2000) that Ca-alginate is not effective in protecting cells in low pH conditions and survival depends on various parameters such as alginate concentration and bead size. Therefore, although Ca-alginate could help protect cells in orange juice, its protective effect after ingestion is questionable. Interestingly, in the study by Ding and Shah

(2008), unprotected cells survived for 4 weeks in orange juice and complete loss in viability was observed by the 5th week. Our study is not in agreement with this, since complete loss in viability of unprotected *B. longum* cells was seen by week 3.

A recent study (Su *et al.*, 2011) used 1.5% (w/v) alginate – 2% (w/v) human-like collagen microspheres to protect *B. longum* cells during storage in 10% (w/v) skimmed milk at 4˚C for 3 weeks. After 3 weeks, the survival of the cells decreased from 9.35 to 7.38 log CFU/ml (a log reduction in viability of 1.97 CFU/ml). When *B. longum* cells were protected with γ-PGA during storage in orange juice for 3 weeks in our study, a lower log reduction in viability (1.30 CFU/ml) was seen. It must also be noted that orange juice has a lower pH than skimmed milk and provides a more unsuitable environment for bacteria, especially for *Bifidobacteria* which are sensitive to pH. It is evident that γ-PGA is more efficient in protecting *B. longum* during storage at 4˚C. In addition, production of microspheres requires the usage of electrostatic droplet generation technology which could have added initial investment implications. Protection of cells with γ-PGA is less complicated.

In another study (Akalin *et al.*, 2004), the prebiotic chicory fructooligosaccharide (FOS) was used to protect *B. longum* and *B. animalis* in yoghurt for 28 days at 4˚C. *B. longum* was seen to be more sensitive to storage conditions than *B. animalis*. After 28 days, the viability of *B. longum* protected with FOS decreased from 4.06 x 10^7 CFU/g to 2 x 10^5 CFU/g (a log reduction in viability of 2.31 CFU/ml). When γ-PGA was used to protect *B. longum* cells during storage in orange juice for 28 days in our study, a log reduction in viability of 1.56 CFU/ml was observed. It must again be noted that the pH of orange juice (3.91) is lower than that of yoghurt (~ 4.5). Thus orange juice is a less suitable environment for *B. longum*

compared to yoghurt. γ-PGA seems to be better at protecting *B. longum* during storage than FOS.

On assessing results obtained from tests conducted with orange juice, it can be said that protecting cells with γ-PGA could make orange juice a potential delivery platform for probiotic bacteria. The effect of various protective measures on the viability of probiotic bacteria in different foodstuffs has been summarized in **Table 7.1**.

Table 7.1: The use of various protective measures to maintain viability of probiotic bacteria during storage in foodstuff

Organism	Foodstuff	Method of protection	Duration	Log CFU/ml reduction in viability	Reference
B. longum	Orange juice	γ-PGA	39 days	2.99	See section 6.4.2.1
B. breve	Orange juice	γ-PGA	39 days	2.05	See section 6.4.2.2
B. longum	Orange juice	Ca-alginate	6 weeks	3.00	(Ding and Shah, 2008)
B. longum	Skimmed milk	Alginate – human-like collagen	3 weeks	1.97	(Su *et al.*, 2011)
B. longum	Yoghurt	FOS	28 days	2.31	(Akalin *et al.*, 2004)
B. bifidum	Stirred yoghurt	Whey protein concentrate	35 days	3.00	(Christopher *et al.*, 2009)

Similarly, the effect of 2.5% (w/v) γ-PGA was tested on the viability of *Bifidobacteria* when stored in pomegranate juice (**Fig 6.7 & 6.8**). *B. longum* cells that were not protected with γ-PGA died within 6 days. γ-PGA-protected *B. longum* cells died within 20 days. However, even on day 6, the viability of the cells was less than 6 log CFU/ml. Unprotected *B. breve* showed complete loss in viability within 2 days, whilst γ-PGA protected-*B. breve* died within 13 days. However, even on day 2, the cells showed less viability than the recommended FAO value (6-7 log CFU/ml). Clearly, *B. breve* cells are more sensitive to pomegranate juice than *B. longum*. The viability of unprotected cells in pomegranate juice was very poor, whilst γ-PGA protected the cells a little better, although this was not good enough to make

pomegranate juice a suitable food for delivery of probiotics. Poor survival of unprotected *B. longum* and *L. plantarum* in pomegranate juice has also been demonstrated in other studies (Nualkaekul and Charalampopoulos, 2011; Nualkaekul *et al.*, 2011), where the viability of the cells decreased by ~8 log CFU/ml within 1 week (*B. longum*) and 4 weeks (*L. plantarum*) of storage at 4˚C. γ-PGA-protected *B. longum* in pomegranate juice survived slightly better in our study.

Pomegranate juice contains a much lower content of ascorbic acid than orange juice. Ascorbic acid is an oxygen scavenger and helps promote anaerobic conditions which are ideal for *Bifidobacteria*. In addition, pomegranate juice contains a high amount of antioxidants and has anti-microbial properties, which has been attributed to its high polyphenol content (Johanningsmeier and Harris, 2011). It has been found that the antioxidant capacity of pomegranate juice is higher than that of orange juice, where the total phenolic content of pomegranate juice is 112.3 mg% in comparison to 22 mg% in orange juice (Parashar and Badal, 2011). Pomegranate and its extracts have been used as natural antimicrobial agents previously (Hayrapetyan *et al.*, 2012; Devi *et al.*, 2011). Hence, these could be the reasons for poor survival of unprotected and γ-PGA-protected probiotic bacteria when they were tested in it. The therapeutically beneficial pomegranate constituents include ellagic acid, punicic acid, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols and flavones (Devi *et al.*, 2011). Addition of probiotic bacteria to pure pomegranate juice would be an added benefit to the health promoting factors already present. However, this study has shown this to be challenging and there is a need to come up with a better protective agent for incorporating probiotic bacteria into pomegranate juice.

To preserve the organoleptic properties and nutritional value of the juice, it was important to identify any changes in the organic acid concentration in the juice on addition of probiotic bacteria and γ-PGA. Ascorbic, citric and malic acid concentrations were monitored for fresh juice and expired orange and pomegranate juice under different conditions (**Tables 6.3, 6.5, 6.7 & 6.9**). The initial concentration of malic acid in fresh orange juice was 3.84 g/l. This concentration did not change considerably for orange juice without probiotic bacteria, orange juice with probiotic bacteria and orange juice with γ-PGA-protected probiotic bacteria. This could be because both probiotic bacteria under study do not preferentially metabolize malic acid. The concentration of malic acid in fresh pomegranate juice did not change significantly on addition of unprotected probiotic and γ-PGA-protected bacteria after 39 days either. Similar results were obtained when *B. longum* were encapsulated with Ca-alginate and stored in orange juice for 6 weeks, where change in malic acid concentration was negligible (Ding and Shah, 2008). Surprisingly, the study by Ding and Shah (2008) did not assess the change in concentrations of ascorbic and citric acid which are important organic acids present in orange juice that contribute to its organoleptic and nutritional properties.

The concentration of ascorbic acid in fresh orange juice in our study was 425.09 mg/l. When fresh orange juice was stored at 4˚C for 39 days, the concentration of ascorbic acid dropped to 160.97 mg/l. This was expected, since ascorbic acid breaks down quickly in the presence of oxygen. The exposure of orange juice to oxygen during routine sampling at regular intervals would have caused the ascorbic acid to break down and reduce in concentration. When *B. longum* was added to orange juice, the concentration of ascorbic acid was seen to reduce further to 79.14 mg/l. Protecting *B. longum* with γ-PGA did not change the concentration of ascorbic acid in orange juice significantly in comparison to orange juice with unprotected *B. longum* after 39 days ($P > 0.05$). The reduction in ascorbic acid content

on addition of *B. longum* could be an independent or combined effect of metabolism or sequestration of ascorbic acid by cells and a physical masking of ascorbic acid by the high molecular weight γ-PGA. In contrast, the addition of unprotected *B. breve* and γ-PGA-protected *B. breve* to orange juice did not significantly alter the ascorbic acid concentration when compared to that in expired orange juice after 39 days ($P > 0.05$). This could probably mean that *B. breve* cells did not metabolize or sequester ascorbic acid. Therefore, the addition of *B. longum* reduced the concentration of ascorbic acid slightly, when compared to expired orange juice without probiotic cells, whereas addition of *B. breve* did not affect the concentration of ascorbic acid. On the other hand, only 9 mg/l of ascorbic acid was seen to be present in fresh pomegranate juice. This is because the pomegranate juice used for the tests is flash-pasteurized and hence, most of the ascorbic acid is destroyed. The juice was not supplemented with ascorbic acid after pasteurization, hence its concentration in fresh juice is very low.

The citric acid concentration in fresh orange juice was found to be 9.95 g/l. The concentration of citric acid in orange juice stored for 39 days at 4˚C was not significantly different from that in fresh orange juice ($P > 0.05$). When *B. longum* and *B. breve* were added to orange juice, the concentration of citric acid was seen to drop slightly (by 0.76 and 0.66 g/l respectively). This could be due to either metabolism or sequestration of citric acid by the cells. The addition of γ-PGA-protected *B. longum* and *B. breve* to orange juice caused an additional slight drop in the citric acid concentration, but was not statistically significant ($P > 0.05$). The reason for the increased drop in citric acid concentration in the presence of γ-PGA could be because probiotic bacteria survive better when protected with γ-PGA, hence, a greater number of viable cells would be available to metabolize and sequester citric acid. In addition, physical masking of a small amount of citric acid by the high molecular weight γ-

PGA could be a possibility. The concentration of citric acid in fresh pomegranate juice (17.43 g/l) did not change significantly after 39 days, even after addition of probiotic bacteria with γ-PGA. This could be attributed to the poor survival of bacteria in pomegranate juice and hence minimal loss in citric acid due to metabolism or sequestration by cells.

The pH of fresh orange juice and orange juice stored at 4˚C for 39 days was 3.91 and 3.81 respectively (**Tables 6.4 & 6.6**). This did not change considerably on addition of unprotected probiotic bacteria. When γ-PGA protected cells were added to orange juice, the pH was seen to increase slightly (by 0.45 and 0.31 for *B. longum* and *B. breve*, respectively). This is due to the addition of the sodium salt of polyglutamate. However, the change in pH was very small. The change in pH was smaller (0.07) when *B. longum* were encapsulated with Ca alginate and added to orange juice which was stored at 4˚C for 6 weeks (Ding and Shah, 2008). A similar trend was observed in pomegranate juice after 39 days (**Tables 6.8 & 6.10**), where an increase in pH of 0.97 and 0.87 was seen when γ-PGA protected *B. longum* and *B. breve*, respectively, were added to the juice.

After performing tests with probiotic bacteria in orange juice and pomegranate juice, it was evident that γ-PGA protects the cells well in orange juice and that orange juice can be a good medium through which probiotic bacteria could be administered. Presently, dairy probiotic products dominate the probiotic food market. This study provides another food range in which probiotic bacteria could be delivered. This would be beneficial, especially for individuals who are lactose intolerant or allergic to milk proteins, and are unable to consume dairy probiotic products. An added advantage of adding low concentration of γ-PGA to fruit juices is that it enhances taste and drinkability of the juice (Yamanaka and Kikuchi, 1991).

7.9 γ-PGA protection of probiotic bacteria in simulated gastric juice

A probiotic food product would be useful only if an adequate number of live probiotic bacteria reach the target site. A heavy loss in viability of probiotic bacteria has been demonstrated in the stomach, which is a region of high acidity (Cui *et al.*, 2000; Su *et al.*, 2011; Vasiljevic and Shah, 2008). Since, γ-PGA has a unique property of staying stable in an acidic environment and disintegrating in a weaker acidic or neutral environment, the protective effect of γ-PGA was tested on the viability of *B. longum* and *B. breve* in a simulated gastric environment (**Fig 6.9 & 6.10**). Unprotected *Bifidobacteria* cells (*B. longum* and *B. breve*) died within 2 h of incubation in simulated gastric fluid. In fact, even after an hour of incubation, the cell count dropped to ~4 log CFU/ml. However, γ-PGA-protected *B. longum* cells showed marginal loss in viability over 4 h in simulated gastric juice. For *B. breve*, the number of cells at 0 h and 4 h was not significantly different ($P > 0.05$). Hence, it can be said that protection by γ-PGA caused little or no loss in viability for probiotic *Bifidobacteria* over a period of 4 h in a simulated gastric environment. This is possibly due to the fact that γ-PGA remains structurally stable in a low pH environment. In a pH stability study (Ho *et al.*, 2006a), strong intra-molecular hydrogen bonding existed in γ-PGA at pH 2.0, where it formed a stable compact α-helix conformation. When pH was increased to 4.09, about 50% of the insoluble α-helix conformations transformed into linear random-coil conformations. When the pH was increased to 6.0, all the intra-molecular hydrogen bonding was disrupted and a complete change in conformation was seen from α-helix to random-coils. This property of Na-γ-PGA was possibly useful in protecting probiotic bacteria in the simulated gastric juice environment with a high acidic pH (pH 2.0). Also, once Na-γ-PGA reaches the intestine, which is a region of weak acidic-neutral pH, the polymer would disintegrate because of the disruption of hydrogen bonds and release the bacteria into the target site.

When 1.5% (w/v) alginate – 2% (w/v) human-like collagen microspheres was used to protect *B. longum* in a simulated gastric juice (pH 2.0) for 2 h, a log reduction in cell viability of 4.66 CFU/ml was observed (Su *et al.*, 2011). This was 4.19 log CFU/ml higher than the reduction in viability observed with γ-PGA in this study, despite the fact that the incubation time was twice as long. It is evident that γ-PGA is much better at protecting cells in simulated gastric juice than alginate – human-like collagen microspheres. In addition, γ-PGA protection of cells does not require the use of any complicated technology to achieve a protective coating of the cells.

In another study (Cui *et al.*, 2000), the survival and stability of *B. bifidum* in simulated gastric juice was assessed after they were loaded in alginate poly-L-lysine microparticles. After 2 h, a 2 log CFU/ml reduction in viability was observed when protected cells were incubated in simulated gastric juice. This reduction in viability was thought to be due to the surface pinholes in microparticles, through which the simulated gastric juice could enter. γ-PGA was much better at protecting *Bifidobacteria* in simulated gastric juice for 4 h, where a maximum log reduction in viability of 0.47 CFU/ml was observed.

Recently, chocolate matrices have also been used as delivery agents for probiotic bacteria (Possemiers *et al.*, 2010). Dark and milk chocolates were evaluated for their effect on the viability of probiotic bacteria during their passage through the stomach and small intestine. Milk chocolate offered better protection (91% and 80% survival for *L. helveticus* and *B. longum*, respectively) than dark chocolate after the cells were passed through simulated gastric and intestinal juices for 3.5 h, and this was thought to be due to the presence of 5-fold lower polyphenol content in milk chocolate. Although milk chocolate protected probiotic bacteria well, γ-PGA-protected *B. longum* survived much better in simulated gastric juice,

where 94.83% survival was seen after 4 h. In addition using milk chocolate as a health promoting food is questionable and it would be particularly unsuitable for diabetic patients. The survival of cells protected with γ-PGA in simulated intestinal juice needs to be tested.

The effect of different protective measures on the viability of probiotic bacteria in simulated gastric juice has been summarized in **Table 7.2**.

Table 7.2: The effect of various protective measures on viability of probiotic bacteria in simulated gastric juice

Organism in simulated gastric juice	Method of protection	Duration	Reduction in viability	Reference
B. longum	γ-PGA	4 h	0.47 log CFU/ml (94.83% survival)	See section 6.4.5.1
B. breve	γ-PGA	4 h	No loss in viability	See section 6.4.5.2
B. longum	Alginate – human-like collagen	2 h	4.66 log CFU/ml	(Su *et al.*, 2011)
B. bifidum	Alginate-poly-*l*-lysine	2 h	2 log CFU/ml	(Cui *et al.*, 2000)
B. longum (sim gastric and intestinal juice)	Milk chocolate	3.5 h	80% survival	(Possemiers *et al.*, 2010)
B. lactis	Maple sap concentrate	1.3 h	0.4 - 1.2 log CFU/ml	(Khalf *et al.*, 2010)

Thus, it is clear that γ-PGA could potentially help in improved survival and viability of probiotic bacteria in the stomach. Once the cells are transported to the intestine, it has been demonstrated that γ-PGA breaks down steadily into its component glutamic acid residues (Singer, 2005). This would release probiotic bacteria into the target site. Glutamic acid can enter normal cellular metabolism and is not excreted by the kidney.

Saarela *et al.* (2006) highlight the importance of choosing a cryoprotectant to protect probiotic bacteria during freeze drying that would also protect them during storage in the appropriate food delivery system (Saarela *et al.*, 2006). Not only does γ-PGA protect bacteria

during freeze drying and storage in orange juice, but it also protects bacteria in simulated gastric juice, hence making it an ideal choice for a delivery tool for protection of probiotic bacteria.

8. CONCLUSION

γ-PGA is a unique biopolymer made up of repeating units of glutamic acid. It is biodegradable, non-toxic and non-immunogenic, and as such, has the potential to be used for multiple applications. It is important to investigate new bacteria for γ-PGA production and to characterize the biopolymer they produce, because this would help deliver a more consistent and better quality polymer for use in different industries.

In this study, γ-PGA production in eight different bacteria was investigated in two media (GS and E). Two of these bacteria (*B. subtilis* natto and *B. licheniformis* 9945a) have been used for γ-PGA production before. The other six bacteria (*B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859, *B. licheniformis* 1525 and *B. licheniformis* 6816) have not been used previously for this purpose.

Overall, it was seen that bacteria grown in GS medium reached a higher cell count (up to 9.58 log CFU/ml) than when they were grown in medium E (up to 8.57 log CFU/ml). This was possibly due to the presence of a vitamin solution in GS medium. In addition, more sucrose was consumed by most of the *B. subtilis* strains when grown in GS medium than glycerol when they were grown in medium E. Conversely, all *B. licheniformis* strains consumed more glycerol in medium E than sucrose in GS medium. It was also found that most of the *B. subtilis* strains consumed more L-glutamic acid when sucrose was used as the carbon source, whereas most *B. licheniformis* strains consumed more L-glutamic when glycerol was used as the carbon source.

The highest yield of γ-PGA in medium E (22.3 g/l) was produced by *B. licheniformis* 1525 and the highest γ-PGA yield in GS medium (17.77 g/l) was produced by *B. subtilis* natto. Three bacteria, *B. subtilis* 23857, *B. subtilis* 23859 and *B. licheniformis* 1525 produced more

γ-PGA (g/l) in medium E than in GS medium. In fact, all bacteria produced more γ-PGA/cell in medium E. This was possibly due to the presence of citric acid and glycerol. It can be concluded that sucrose (GS medium) improved cell growth whereas presence of glycerol and citric acid (medium E) increased γ-PGA production. For *B. subtilis* natto, GS medium was more suited for L-glutamic acid consumption and total γ-PGA production than medium E, since it utilized the maximum amount of L-glutamic acid (19 g/l) and produced the highest γ-PGA yield (17.77 g/l). When compared to other bacteria, *B. subtilis* natto utilized the least L-glutamic acid (4.66 g/l) and produced the lowest yield of γ-PGA (5.7 g/l) in medium E.

The properties of the γ-PGA produced are crucial, since they can determine the application for which it is used. γ-PGA that was produced by bacteria grown in GS medium was white in colour, whereas cells produced a brown polymer when grown in medium E. The colour of the polymer is important since a white polymer is ideal for medical and food applications whereas a brown polymer could be used for applications in the waste water treatment industry.

All bacteria produced γ-PGA, which was confirmed by FT-IR analysis. It was found that crystallinity and form of γ-PGA were dependent on the medium of γ-PGA production and not on the bacteria producing it, since all bacteria produced a crystalline, Na-salt form of γ-PGA when grown in GS medium and an amorphous acid form of γ-PGA when grown in medium E. This is the first report that establishes a dependence of crystallinity and form of γ-PGA on the medium of production.

Knowledge of molecular weight of γ-PGA is extremely crucial before it is used for an application. Eight bacteria grown in two different media produced γ-PGA with molecular

weights ranging from ~3000 Da to 871,000 Da. A low molecular weight γ-PGA is especially important for medical applications whereas a high molecular weight polymer can be used for applications in the cosmetic and waste water treatment industry. Unlike crystallinity and form of γ-PGA (which were only dependent on the medium), molecular weight of the produced polymer was seen to be dependent on both bacteria and medium used for its production

After characterization studies of γ-PGA produced by the eight bacteria in both media, the best γ-PGA producer was selected for subsequent investigations. *B. subtilis* natto grown in GS medium was chosen to produce γ-PGA for testing novel probiotic applications. Production of γ-PGA from the bacterium was scaled up by growing it in a 5 l fermenter to increase the yield of γ-PGA produced as well as the total quantity of γ-PGA obtained. On growing bacteria in a fermenter, where parameters such as temperature, pH, dO_2, aeration and agitation could be monitored and controlled, cell counts of up to 10.11 log CFU/ml and γ-PGA yield of up to 27.94 g/l were achieved. Before the γ-PGA obtained from fermentations was used for probiotic tests, different methods of sterilization of γ-PGA were tested. There is no report on sterilization of γ-PGA in the literature. On assessing different techniques, autoclaving γ-PGA at 110˚C and 0.35 BAR for 30 mins was found to be the best method, because it completely eliminated residual bacteria in γ-PGA without disrupting the structural integrity of the polymer.

Bacteria are usually freeze dried to lengthen storage time. However, the freeze drying process itself is detrimental to the viability of bacteria. Although the antifreeze activity of γ-PGA has been assessed previously, it has never been used to maintain viability of probiotic bacteria during freeze drying. Three probiotic bacteria (*L. paracasei*, *B. longum* & *B. breve*) were protected with sterilized and untreated γ-PGA along with sucrose (which is commonly used

as a cryoprotectant commercially) and the viability before and after freeze drying was determined. It was found that *L. paracasei* was more resistant to the freeze drying process than the *Bifidobacteria* strains, because a 1.34 log CFU/ml reduction in viability was seen for *L. paracasei* that were not protected with a cryoprotectant in contrast to 2.47-2.52 log CFU/ml reduction in viability for unprotected *Bifidobacteria*. Also, for *L. paracasei*, 10% γ-PGA[S] offered significantly better protection ($P < 0.05$) than 10% sucrose. The cryoprotectant activities of 5% γ-PGA[S] and 10% sucrose were comparable ($P > 0.05$). For *Bifidobacteria*, 10% γ-PGA[S] and 10% sucrose were equally efficient in maintaining viability during freeze drying. 5% γ-PGA (S) and 10% γ-PGA[U] were not as effective as 10% γ-PGA[S].

Most probiotic bacteria in foods are delivered via dairy products such as milk, yoghurts and yoghurt drinks. There is a need to develop non-dairy vehicles for administration of probiotics for individuals who are lactose intolerant or who are allergic to milk proteins. Fruit juices are good candidates for this purpose, since they are rich in nutrients and are palatable. However, probiotic viability in fruit juice is hampered due to factors such as pH, storage temperature, oxygen levels, naturally present antimicrobials and/or presence of competing microorganisms. Hence, it is important to protect the viability of probiotic bacteria in such environments. γ-PGA was utilized for this novel application where it was shown to protect *B. breve* and *B. longum* in orange juice for 39 days at 4˚C. Cells that were not protected with the polymer showed complete loss in viability within 20 days. Even on day 11, the number of unprotected cells in orange juice was much less than the recommended FAO value of 6-7 log CFU/ml. Cells protected with γ-PGA survived well for 39 days with a log reduction in viability of less than 2.99 CFU/ml. γ-PGA was not effective in protecting probiotic bacteria in pomegranate juice, since the viability of unprotected and γ-PGA-protected *Bifidobacteria*

was poor in the juice, possibly due to the presence of a high amount of antioxidants and anti-microbial agents.

The change in concentration of organic acids and pH was also determined to assess any variation in the organoleptic and nutritional value of fruit juices. No considerable change was observed in the concentrations of citric acid, malic acid and ascorbic acid when probiotic bacteria and γ-PGA were introduced into orange juice. The pH of orange juice also did not change greatly on addition of γ-PGA-protected *Bifidobacteria*. Overall, it can be said that addition of γ-PGA-protected *Bifidobacteria* did not seem to change the organoleptic and nutritional properties of orange juice substantially. γ-PGA protection was shown to improve the viability of probiotic bacteria in orange juice and hence, it could be used as a non-dairy delivery platform for these bacteria.

Because of the harsh conditions of the stomach, a heavy loss in viability is seen when probiotic bacteria are ingested. Due to its unique ability to remain stable in low pH environments and to disintegrate in a weaker acidic or neutral environment, γ-PGA can be used to improve the survival of these bacteria in the stomach. This was demonstrated by protecting *B. longum* and *B. breve* with γ-PGA and checking their viability at regular intervals when stored in a simulated gastric juice (pH 2.0) for 4 h. It was seen that unprotected cells showed poor viability (~4 log CFU/ml) after 1 h of incubation in simulated gastric juice and showed complete loss in viability within 2 h. On the other hand, only a marginal loss in viability was seen for γ-PGA-protected *B. longum* after 4 h in simulated gastric juice. For *B. breve*, cell viability at 0 h and 4 h was not significantly different ($P > 0.05$). γ-PGA was successful in protecting *Bifidobacteria* in simulated gastric juice with little or no loss in viability.

While choosing an agent to protect probiotic bacteria, it is essential to choose the one that protects them during different stages where loss in viability can be anticipated. The results obtained from our study demonstrates that not only does γ-PGA protect bacteria during freeze drying and storage in orange juice, but it also protects bacteria in simulated gastric juice, hence making it an ideal choice to protect probiotic bacteria during storage and after ingestion.

It can be concluded that this study has increased the information about γ-PGA production in bacteria by characterizing six bacterial strains that had not previously been investigated for this purpose. Each strain produced γ-PGA with different properties in the two media used in this study. γ-PGA was also successfully used for novel probiotic applications, where it improved survival of three probiotic bacteria during freeze drying. This study also showed that a non-dairy food product, orange juice, could be successfully used to deliver *Bifidobacteria* when they were protected with γ-PGA. *Bifidobacteria* survived in orange juice well at 4˚C after 39 days and the viability of *Bifidobacteria* in orange juice was higher than the minimum viability (6-7 log CFU/ml) recommended by FAO. This would be particularly beneficial for individuals who are lactose intolerant or are allergic to milk proteins. Finally, this study showed that γ-PGA could be used to protect *Bifidobacteria* in simulated gastric juice (pH 2.0) at 37˚C for 4 h with very little or no loss in viability. Therefore, γ-PGA can potentially be used to protect probiotic bacteria in the stomach and eventually deliver them to the intestine. γ-PGA would be broken down into component glutamic acid residues and absorbed, presenting no residual effect on the host.

9. FUTURE WORK

The results obtained in this study open up interesting opportunities for future work. For instance, nutrient consumption analysis revealed that when *B. subtilis* natto was grown in medium E for production of γ-PGA, the L-glutamic acid concentration in the medium increased after 72 h. This could indicate the presence of a γ-PGA degradation system, which is active in some bacteria in the late stationary phase and can break down the polymer, thus releasing L-glutamic acid units into the medium. The presence of the components of this system could be investigated further for *B. subtilis* natto grown in medium E. It was also found that *B. subtilis* natto produced only 5.7 g/l of γ-PGA in medium E, which was the lowest among all bacteria grown in this medium. This could be attributed to the presence of the γ-PGA degradation system, which seemed to be active after 72 h. Therefore, it would be interesting to extract γ-PGA from the medium at an earlier time point and check whether this improves the yield of γ-PGA produced.

It was noted that L-glutamic acid was not consumed completely by the cells. This was especially true for *B. licheniformis* strains in GS medium and most bacteria in medium E. It would also be interesting to assess the roles of L-glutamic acid, citric acid and carbon source used in γ-PGA production for bacteria under study. L-glutamic acid could be completely eliminated from the medium and replaced with metabolic precursors such as L-glutamine to check whether the bacteria under study still produce γ-PGA. Optimization of the amount of L-glutamic acid needed to produce γ-PGA for each bacterium would help reduce the cost of γ-PGA production.

It was observed that for most strains in our study, the sucrose concentration remained stable once cells started dying in culture. This could also indicate that sucrose is utilized by the cells

as the growth-limiting substrate. This could be confirmed by determining the fraction of γ-PGA repeat units that were formed from exogenously supplied sucrose.

It was seen that bacteria used to produce γ-PGA reached lower cell numbers in medium E than they did in GS medium. This could be due to the presence of MS vitamin solution in GS medium and/or a very high concentration of glycerol in medium E. A recent study shows that a very high concentration of glycerol (80 g/l), such as that used in our study, inhibits cell growth in *B. subtilis* NX-2 and hence it has been suggested that glycerol could be added later (for example, at ~16 h) to reduce the initial inhibitory effect on cell growth (Wu *et al.*, 2010a). This technique could be used for increased cell growth in medium E and it might be able to further increase the yield of γ-PGA produced in this medium.

This study has investigated the production of γ-PGA in eight bacteria using medium E. In another study (Du *et al.*, 2005), when *B. licheniformis* WBL-3 was used for the production of γ-PGA in modified medium E (using different concentration of glycerol) in shake flasks, a maximum yield of 16.7 g/l was obtained. Four of the eight strains in our study produced γ-PGA with yields higher than 19 g/l in medium E without any modifications or optimization. It would be worth optimizing the production of γ-PGA with respect to carbon source and nitrogen source for these bacteria in fermenters, where better monitoring and control of nutrients is possible. Optimization using a statistical approach has proved beneficial to improve the yield of γ-PGA previously (Bajaj and Singhal, 2009b; Bajaj *et al.*, 2009; Jeong *et al.*, 2010) and could be used for optimizing yield of γ-PGA produced by bacteria in future. Other factors in the medium that can affect the yield of γ-PGA production by each bacterium (such as pH, salt concentration, addition of precursors etc.) would also need to be investigated.

When bacteria were grown in medium E, it was found that the γ-PGA produced was a combination of acid form and sodium salt form of the polymer. Because 3 M NaOH was used to adjust the pH of medium E, production of a pure acid form of γ-PGA from this medium will be attempted by completely eliminating sodium from the medium by adjusting the pH with NH_4OH instead of NaOH.

γ-PGA is an extracellular product. On the other hand, polyhydroxyalkanoates (PHA's) are known to be produced by *Bacillus* strains as intracellular granules (Verlinden *et al.*, 2007). After production of γ-PGA, the cells are discarded since the polymer is released into the medium. It will be interesting to investigate whether the bacterial strains used to produce γ-PGA in our study also produce intracellular PHA's in GS or E media. If this is possible, it could make the process of production of all these useful polymers more economical.

Aqueous hydrolysis of γ-PGA has been performed previously by heating an aqueous solution of γ-PGA at different temperatures to reduce its molecular weight (Goto and Kunioka, 1992). In this work, γ-PGA produced by *B. subtilis* natto in GS medium was sterilized by autoclaving at 110˚C and 0.35 BAR for 30 mins. γ-PGA with low molecular weight has been shown to have higher antifreeze activity than a high molecular weight polymer (Mitsuiki *et al.*, 1998; Shih *et al.*, 2003) and this could be the reason why 10% γ-PGA[S] was found to be a better cryoprotectant for probiotic bacteria than 10% γ-PGA[U] in our study. FT-IR analysis revealed that the structural integrity of the polymer remained intact after sterilization. However, GPC analysis could be used to determine the reduction in molecular weight of the polymer after autoclaving.

γ-PGA was successful in protecting cells during freeze drying, storage in orange juice and incubation in simulated gastric juice. It is crucial to reduce the added cost of γ-PGA protection as much as possible so that this application of γ-PGA is feasible for use in industry. Different concentrations of γ-PGA were tested for their efficiency of protection and it was found that 10% γ-PGA could protect cells much better than 5% γ-PGA during freeze drying. SEM images of γ-PGA-protected *Bifidobacteria* revealed that some γ-PGA was still available to coat bacteria. Therefore, it would be interesting to increase the number of cells to be protected with 10% γ-PGA and check their survival during freeze drying. This might help in protecting more bacteria with the same amount of γ-PGA. Lowering the ratio of γ-PGA to cells could make this novel application commercially viable.

Comparing the cryoprotectant test results of our study to other studies (Jagannath *et al.*, 2010; Siaterlis *et al.*, 2009) indicates that γ-PGA could offer better protection to *Lactobacilli* during freeze drying than trehalose, sorbitol and *nata*. This needs to be confirmed by performing additional tests.

It would be useful to check the viability of freeze-dried bacteria protected with γ-PGA, when stored at different temperatures (for example, 4˚C, 25˚C and 37˚C) for a longer duration (2 to 6 months) to test the long-term stability of γ-PGA-protected bacteria. This would be useful, since refrigeration for storage of bacteria is not readily available in some parts of the world and long term protection of bacteria at the ambient temperatures found in markets such as Asia and Africa could be beneficial.

This study demonstrated that γ-PGA was successful in protecting the cells when they were introduced in orange juice and stored for 39 days at 4˚C, but it was not effective in protecting

the cells in pomegranate juice. Other fruit juices, such as grapefruit, pineapple, blackcurrant, strawberry, cranberry, apple, mango, lychee and guava also have nutritional value and the protective effect of γ-PGA on probiotic bacteria could be tested in these juices as well.

γ-PGA was very successful in protecting the viability of probiotic bacteria in simulated gastric juice, where little or no loss in viability was observed for 4 h. Next, the protective effect of γ-PGA on probiotic bacteria will be tested when they are stored in a simulated intestinal juice and when they pass through an artificial human gut model. Using Ca-γ-PGA (instead of Na-γ-PGA) for protection of probiotic bacteria through the gastrointestinal tract could be beneficial, because it has been shown that γ-PGA can increase the calcium absorption in the intestine (Tanimoto *et al.*, 2007). This would also be beneficial for delivering probiotic bacteria via a non-dairy product such as fruit juice, since the calcium usually derived from dairy sources could be obtained from Ca-γ-PGA in the juice. Therefore, it would be interesting to assess the protective effect of Ca-γ-PGA on probiotic bacteria under different conditions.

Vitamin B12 (Cobalamin) is a water-soluble vitamin and plays a key role in the normal functioning of the brain and nervous system. Deficiency of vitamin B12 can potentially cause several disorders such as megaloblastic anaemia and vitamin B12 deficiency-associated neuropathy. The classic treatment for vitamin B12 deficiency is parenteral administration, however, new routes of vitamin B12 administration (oral and nasal) are being investigated. Oral administration of vitamin B12 is challenging, because of its instability in the gastrointestinal tract. In an earlier study (Sonaje *et al.*, 2010), nanoparticles comprising chitosan and γ-PGA have been used for effective oral delivery of insulin. The protective

effect of these nanoparticles on vitamin B12 after ingestion could also be tested, which could help reduce cost of administration and injection related complications.

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11. APPENDICES

Table 11.1: Two-factor ANOVA for 3 probiotic bacteria freeze dried using 10% γ-PGA[S], 5% γ-PGA[S] and 10% sucrose. Control represents cells that were freeze dried without any cryoprotectant.

Before freeze drying vs Control	t	P Value	Summary
B. breve	36.61	P<0.001	***
B. longum	37.29	P<0.001	***
L. paracasei	18.88	P<0.001	***
Before freeze drying vs 10% Sucrose			
B. breve	18.84	P<0.001	***
B. longum	17.46	P<0.001	***
L. paracasei	13.52	P<0.001	***
Before freeze drying vs 10% γ-PGA[S]			
B. breve	19.83	P<0.001	***
B. longum	19.30	P<0.001	***
L. paracasei	8.693	P<0.001	***
Before freeze drying vs 5% γ-PGA[S]			
B. breve	31.05	P<0.001	***
B. longum	32.12	P<0.001	***
L. paracasei	15.17	P<0.001	***
Control vs 10% Sucrose			
B. breve	17.77	P<0.001	***
B. longum	19.83	P<0.001	***
L. paracasei	5.368	P<0.001	***
Control vs 10% γ-PGA[S]			
B. breve	16.77	P<0.001	***
B. longum	17.99	P<0.001	***
L. paracasei	10.19	P<0.001	***
Control vs 5% γ-PGA[S]			
B. breve	5.557	P<0.001	***
B. longum	5.172	P<0.001	***
L. paracasei	3.715	P<0.01	**
10% Sucrose vs 10% γ- PGA(S)			
B. breve	0.9945	P > 0.05	ns
B. longum	1.841	P > 0.05	ns
L. paracasei	4.824	P<0.001	***
10% Sucrose vs 5% γ-PGA[S]			
B. breve	12.21	P<0.001	***
B. longum	14.66	P<0.001	***
L. paracasei	1.652	P > 0.05	ns
10% PGA(S) vs 5% γ-PGA[S]			
B. breve	11.22	P<0.001	***
B. longum	12.82	P<0.001	***
L. paracasei	6.476	P<0.001	***

Table 11.2: Two-factor ANOVA for change in organic acid concentration in orange juice (OJ) when free and γ-PGA-protected probiotic bacteria were added to it and stored at 4˚C for 39 days. (Exp = Expired; Con = Control [Unprotected cells]; Test = γ-PGA-protected cells).

Organic acid	t	P value	Summary
Fresh OJ vs Exp OJ			
Ascorbic acid concentration mg/l	18.73	P<0.001	***
Citric acid concentration g/l	0.01362	P > 0.05	ns
Malic acid concentration g/l	0.007748	P > 0.05	ns
Fresh OJ vs *Breve* OJ Con			
Ascorbic acid concentration mg/l	17.86	P<0.001	***
Citric acid concentration g/l	0.04659	P > 0.05	ns
Malic acid concentration g/l	0.005830	P > 0.05	ns
Fresh OJ vs *Breve* OJ Test			
Organic acid	t	P value	Summary
Ascorbic acid concentration mg/l	16.41	P<0.001	***
Citric acid concentration g/l	0.1157	P > 0.05	ns
Malic acid concentration g/l	0.02886	P > 0.05	ns
Fresh OJ vs *Longum* OJ Con			
Ascorbic acid concentration mg/l	24.53	P<0.001	***
Citric acid concentration g/l	0.05308	P > 0.05	ns
Malic acid concentration g/l	0.01210	P > 0.05	ns
Fresh OJ vs *Longum* OJ Test			
Ascorbic acid concentration mg/l	25.08	P<0.001	***
Citric acid concentration g/l	0.08222	P > 0.05	ns
Malic acid concentration g/l	0.01472	P > 0.05	ns
Exp OJ vs *Breve* OJ Con			
Ascorbic acid concentration mg/l	0.8699	P > 0.05	ns
Citric acid concentration g/l	0.03297	P > 0.05	ns
Malic acid concentration g/l	0.001918	P > 0.05	ns
Exp OJ vs *Breve* OJ Test			
Ascorbic acid concentration mg/l	2.326	P > 0.05	ns
Citric acid concentration g/l	0.1021	P > 0.05	ns
Malic acid concentration g/l	0.03661	P > 0.05	ns
Exp OJ vs *Longum* OJ Con			
Ascorbic acid concentration mg/l	5.803	P<0.001	***
Citric acid concentration g/l	0.03946	P > 0.05	ns
Malic acid concentration g/l	0.004351	P > 0.05	ns
Exp OJ vs *Longum* OJ Test			
Ascorbic acid concentration mg/l	6.344	P<0.001	***
Citric acid concentration g/l	0.06861	P > 0.05	ns
Malic acid concentration g/l	0.02247	P > 0.05	ns
***Breve* OJ Con vs *Breve* OJ Test**			
Ascorbic acid concentration mg/l	1.456	P > 0.05	ns
Citric acid concentration g/l	0.06912	P > 0.05	ns
Malic acid concentration g/l	0.03469	P > 0.05	ns
***Longum* OJ Con vs *Longum* OJ Test**			
Ascorbic acid concentration mg/l	0.5408	P > 0.05	ns
Citric acid concentration g/l	0.02914	P > 0.05	ns
Malic acid concentration g/l	0.02682	P > 0.05	ns

Table 11.3: Two-factor ANOVA for change in organic acid concentration in pomegranate juice (PJ) when unprotected and γ-PGA-protected probiotic bacteria were added to it and stored at 4˚C for 39 days. (Exp = Expired; Con = Control [Unprotected cells]; Test = γ-PGA-protected cells).

Organic acid	t	P value	Summary
Fresh PJ vs Exp PJ			
Ascorbic acid concentration mg/l	1.328	P > 0.05	ns
Citric acid concentration g/l	0.1183	P > 0.05	ns
Malic acid concentration g/l	1.107	P > 0.05	ns
Fresh PJ vs *Breve* PJ Con			
Ascorbic acid concentration mg/l	2.391	P > 0.05	ns
Citric acid concentration g/l	0.7395	P > 0.05	ns
Malic acid concentration g/l	0.4063	P > 0.05	ns
Fresh PJ vs *Breve* PJ Test			
Ascorbic acid concentration mg/l	1.028	P > 0.05	ns
Citric acid concentration g/l	1.873	P > 0.05	ns
Malic acid concentration g/l	0.2630	P > 0.05	ns
Fresh PJ vs *Longum* PJ Con			
Ascorbic acid concentration mg/l	4.277	P<0.001	***
Citric acid concentration g/l	1.046	P > 0.05	ns
Malic acid concentration g/l	1.003	P > 0.05	ns
Fresh PJ vs *Longum* PJ Test			
Ascorbic acid concentration mg/l	9.172	P<0.001	***
Citric acid concentration g/l	0.2265	P > 0.05	ns
Malic acid concentration g/l	0.6206	P > 0.05	ns
Exp PJ vs *Breve* PJ Con			
Ascorbic acid concentration mg/l	1.064	P > 0.05	ns
Citric acid concentration g/l	0.6212	P > 0.05	ns
Malic acid concentration g/l	0.7002	P > 0.05	ns
Exp PJ vs *Breve* PJ Test			
Ascorbic acid concentration mg/l	2.355	P > 0.05	ns
Citric acid concentration g/l	1.754	P > 0.05	ns
Malic acid concentration g/l	1.370	P > 0.05	ns
Exp PJ vs *Longum* PJ Con			
Ascorbic acid concentration mg/l	2.949	P < 0.05	*
Citric acid concentration g/l	1.164	P > 0.05	ns
Malic acid concentration g/l	0.1036	P > 0.05	ns
Exp PJ vs *Longum* PJ Test			
Ascorbic acid concentration mg/l	7.844	P<0.001	***
Citric acid concentration g/l	0.3447	P > 0.05	ns
Malic acid concentration g/l	0.4859	P > 0.05	ns
***Breve* PJ Con vs *Breve* PJ Test**			
Ascorbic acid concentration mg/l	3.419	P<0.01	**
Citric acid concentration g/l	1.133	P > 0.05	ns
Malic acid concentration g/l	0.6693	P > 0.05	ns
***Longum* PJ Con vs *Longum* PJ Test**			
Ascorbic acid concentration mg/l	4.895	P<0.001	***
Citric acid concentration g/l	0.8195	P > 0.05	ns
Malic acid concentration g/l	0.3823	P > 0.05	ns

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Request for grant of a patent

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NP10 8QQ

Application number

1.	Your reference	**TG59959P.GBA**
2.	Full name, address and postcode of the applicant or of each applicant Patents ADP number *(if you know it)*	**University of Wolverhampton** **Wulfruna Street** **Wolverhampton WV1 1LY** **West Midlands** **United Kingdom**
3.	Title of the invention	**IMPROVED VIABILITY OF PROBIOTIC MICROORGANISMS**
4.	Name of your agent *(if you have one)* "Address for service" to which all correspondence should be sent. This may be in the European Economic area or Channel Islands (see warning note below) *(including the postcode)* Patents ADP number *(if you know it)*	**Barker Brettell LLP** **Barker Brettell LLP** **100** **Hagley Road** **Edgbaston** **Birmingham B16 8QQ** **West Midlands** **United Kingdom** **10220036001**
5.	Priority declaration: Are you claiming priority from one or more earlier-filed patent applications? If so, please give details of the application(s)	

Country	Application number	Date of filing	Date available on PDAS

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7.	Inventorship: (Inventors must be individuals not companies) Are all the applicants named above also inventors?	**No**	
8.	Are you paying the application fee with this form?	**Yes**	

Fig 11.1: Patent filed on 02-09-2011 for "Improved viability of probiotic microorganisms".

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New Polymer Research Could Boost Probiotics Industry

ScienceDaily (Sep. 5, 2011) — A protective delivery vehicle that shuttles friendly bacteria safely through the stomach to the intestines could provide a major boost for the probiotics industry. The new technology could also be used for the delivery of certain drugs and even increase calcium absorption, according to research presented at the Society for General Microbiology's Autumn Conference at the University of York this week.

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The probiotic industry is worth £200 million a year in the UK. Probiotic foods contain live beneficial bacteria and may help maintain and improve gut health, strengthen immunity, fight gastro-intestinal and respiratory disorders and even show anti-tumour effects. One of the challenges for manufacturers of probiotic foods is getting high enough numbers of these bacteria into the intestines; most perish under the heavy acidic conditions of the stomach. Scientists from the University of Wolverhampton led by Dr Iza Radecka, have now found a solution to this problem by developing a special type of biopolymer that protects probiotic bacteria in the stomach and delivers them safely to the intestines where they can get to work.

The novel biopolymer is completely biodegradable and is able to remain intact in the stomach and continue to the intestine, where it disintegrates, releasing the bacteria. The researchers showed that beneficial bacteria including Lactobacillus and Bifidobacteria strains were able to survive in a simulated gastric juice solution for up to four hours when they were coated with the polymer. Bacteria that did not have this coating only survived for two hours. "Our research uses a novel biodegradable, edible and non-toxic biopolymer to protect bacteria during storage and after ingestion so that consistent numbers of live and viable friendly bacteria can be administered via food products," explained Dr Radecka.

The researchers believe their findings could have a major impact on the probiotics industry. "There is an ongoing debate about the usefulness of probiotics. Some data showing positive effects is irreproducible and one of the reasons for this could be insufficient numbers of live bacteria reaching the intestine. A product that delivers a consistent number of bacteria to the intestine is therefore essential," said Aditya Bhat, who is carrying out the research and is presenting the group's work. "This will hopefully lead to better quality probiotic food products that can be used to prevent or control gastro-intestinal, dental or respiratory disorders."

The new biopolymer also has the potential for clinical applications outside of the probiotics industry, suggested Aditya. "A variation of this polymer can be used to increase calcium absorption in the intestine that would help maintain healthy bone structure and condition. Also, it looks feasible for the polymer to be used for administering unstable drugs that disintegrate in the gastro-intestinal tract," he said.

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Fig 11.2: Press release in Science Daily (05/09/2011)

Biopolymer protection may be boost to probiotic industry

By Nathan Gray, 06-Sep-2011 Post a comment

Related topics: Probiotics, Research, Probiotics and prebiotics, Gut health

A new biopolymer technology that protects probiotic bacteria during transit through the body could provide a major boost for the industry, according to new research.

The research, due to be presented today at the UK's Society for General Microbiology's autumn conference, reports on a new biopolymer delivery vehicle for the delivery of probiotic bacteria to the gut.

The researchers, led by Dr Iza Radecka from the University of Wolverhampton, noted that one of the challenges for manufacturers of probiotic foods is getting high enough numbers of these bacteria into the intestines, because most perish during processing, storage, and under the heavy acidic conditions of the stomach.

"We thought, if we could coat probiotic bacteria with this, then they could perhaps better survive the harsh environment of the stomach," said Radecka.

"Our research uses a novel biodegradable, edible and non-toxic biopolymer to protect bacteria during storage and after ingestion so that consistent numbers of live and viable friendly bacteria can be administered via food products," she explained.

Stability issue

Radecka told NutraIngredients that there are currently problems with the stability of probiotic populations in products because of the large losses of viable population seen during freeze drying of products, storage time, and during the low pH conditions of the stomach.

"By coating the bacteria with this polymer, we found that the survival at these three steps is fantastic – we can have nearly the same number of bacteria at the end of the process as the number at the beginning," said Radecka.

She explained that the structure of the new biopolymer is very stable at lower pH values, but becomes more relaxed as pH increases to values of around 6 or 7.

The researcher team said that new biodegradable polymer is able to remain intact in the stomach and continue to the intestine, where it disintegrates, releasing the bacteria.

Initial studies have shown that probiotics including Lactobacillus and Bifidobacteria strains were able to survive in a simulated gastric juice solution for up to four hours when they were coated with the polymer – compared to only two hours survival for non-coated populations.

Industry boost

According to Euromonitor International, the value of the global probiotic market, including yoghurt, supplements and juice, was over $20bn (€14.2bn) in 2008, whilst the European probiotic yoghurt market alone was said to be worth $6.73bn (€4.8bn).

The researchers said that they are looking to now perform *in vivo* research on the human digestive system, whilst working on ways to improve the production and costs of the biopolymer vehicle.

"From a health point of view it would be nice to deliver a better amount of probiotic bacteria to the gut, and keep the gut healthy as we known probiotic bacteria can," said Radecka.

"If we an keep the maximum number of cells alive to deliver to the gut then I am sure we can improve human health," she added.

Fig 11.3: Press release in Nutraingredients.com (06/09/2011)

THE HINDU

Health

Breakthrough in shuttling beneficial bacteria through stomach acids

IANS

The Hindu A technician at work in the Yakult Danone factory near New Delhi, India's first establishment for probiotic food. File photo

Transporting friendly bugs to intestines is a challenging task as most of them perish under heavy acidic conditions of the stomach but a new technology can now deliver them safely to the guts.

Probiotic food contains live bugs that help maintain and improve gut health, strengthen immunity, fight gastro-intestinal and respiratory disorders and even show anti-tumour effects.

Scientists at the University of Wolverhampton, Britain, led by Iza Radecka have now developed a biopolymer that delivers the good bugs safely to the intestines where they can get to work.

The new technology could also be used to increase calcium absorption.

The biopolymer is completely biodegradable and is able to remain intact in the stomach and continue to stay in the intestine, where it releases these bugs, according to a Wolverhamption statement.

Good bugs like lactobacillus and bifidobacteria strains were able to survive in a simulated gastric juice solution for up to four hours when they were coated with the biopolymer. Bacteria without this coating only survived for two hours.

"Our research uses a novel biodegradable, edible and non-toxic biopolymer to protect bacteria during storage and after ingestion," explained Ms. Radecka.

Keywords: probiotics, beneficial bacteria, biopolymers

Fig 11.4: Press release in The Hindu, India (06/09/2011)